Biomarkers of mineral dust induced lung disorders : molecular epidemiologic studies in coal workers

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Biomarkers of Mineral Dust Induced Lung Disorders

Molecular epidemiologic studies in coal workers

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. M.J. Cohen,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op woensdag 30 oktober 1996 om 16.00 uur

door

Peter Frans Hoeland Schins

geboren te Heerlen op 27 juli 1966

@ Schins, Maastricht 1996

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Abbreviations

AM	Alveolar macrophage
CI	Confidence interval
CWP	Coal workers' pneumoconiosis
COPD	Chronic obstructive pulmonary disease
dG	2'-deoxyguanosine
DLCO	Diffusion capacity
ECD	Electrochemical detection
ELISA	Enzyme-linked immunosorbent assay
f ₀	Resonant frequency
FCS	Foetal calf serum
FEV ₁	Forced expiratory volume in first second
FN	Fibronectin
FVC	Forced vital capacity
Gpx	Glutathione peroxidase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
GST	Glutathione-S-transferase
GR	Glutathione reductase
Hb	Haemoglobin
HBSS	Hanks' balanced salt solution
HPLC	High performance liquid chromatography
HRCT	High resolution computed tomography
IFN	Interferon- γ
IGF	Insulin like growth factor
IL	Interleukin
ILO	International Labour Office
LPS	Lipopolysaccharide
LTB ₄	Leukotriene-B ₄
MIP	Macrophage inflammatory protein
8-oxodG	7-hydro-8-oxo-2'-deoxyguanosine
PIIIP	Type III procollagen peptide
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PGE	Prostaglandin-E
PMF	Progressive massive fibrosis
RBC	Red blood cells
RIA	Radioimmunoassay
ROC	Receiver operator characteristic curve
ROS	Reactive oxygen species
RR	Relative risk
SD	Standard deviation
SE(M)	Standard error (of mean)
SOD	Superoxide dismutase
TGF β	Transforming growth factor- β
TRAP	Total radical-trapping antioxidant parameter
TX	Thromboxane
TNF	Tumor necrosis factor- α
sTNF-R	Soluble tumor necrosis factor- α receptor

GENERAL INTRODUCTION AND AIMS OF THE STUDY

1.1 Minerals and health effects

Already in the ancient history man realized that the earth crust was rolling in minerals and ores to create tools, pottery and ceramics, or to construct buildings. Remains of stone buildings found near Jericho date back to 9000 B. C. and in Syria tools and ceramics have been excavated originating from Ugarit (6600 B.C.) and Mari (4 500 B.C.) cultures. Egyptians became famous for their sculptured statues and reliefs, and their skilfulness to create beautiful objects of glass, enamel, and metals including gold. The earliest mining activities in the Netherlands date back to 3000 B.C., when flint was exploited and processed in the Southern part of the province Limburg (near the village of St. Geertruid).

Among the various minerals on earth, silica (SiO_2) is the most abundant. Other minerals include limestone (e.g. marble, dolomite), coal (e.g. anthracite, graphite), asbestos fibers, and clay (e.g. kaolin or china clay, mica). Silica is present in granite, feldspar, sandstone, and shale. Shale is often found in the vicinity of coal deposits, and consists of silica, kaolinite, mica and may occasionally contain oil. Natural silica may occur as α -quartz, cristoballite and tridymite. Diatomaceous earth (amorphous silica) calcinated at 800°C to 1000°C is known as kieselguhr. Limestone is used for the production of cement and calcium(hydr)oxide and clays are principal resources for ceramic and pottery industry. Asbestos is a common name for naturally occurring mineral silicate fibers, which have been used extensively because of their great strength and heat resistance. Examples include chrysotile (serpentine fibers), amosite, crocidolite, anthophyllite, tremolite, and actinolite. Naturally occurring non-asbestos fibers include erionite (zeolite), attapulgite, wollastonite, sepiolite and graphite. Man-made fibers may be subdivided into mineral fibers (e.g. glassfibers, rockwool, and slagwool and ceramic fibers), and synthetic fibers (e.g. polyolefin fibers).

Despite the world-wide diversity in abundance of mineral-rich areas and in the structure, physio-chemical properties and related applications of these different minerals, they all share the probability to become airborne as dusts and eventually inhaled. Dusts are defined as small, solid particles formed by the mechanical attrition or disintegration of larger masses of material [Cotes et al., 1987]. In addition to the minerals described above, dust can be generated from metal sources (e.g. iron, tin). Examples of organic dusts are wood-, cotton-, grain- and flour-dust, and the (synthetic organic) aramid fibers.

Deposition of inhaled particles in the lung is primarily dependent on size, shape and particle density, as well as on individual lung morphology (e.g. lung volume) and physiology (breathing rate and depth) [Bohning and Lippmann,

1992]. Generally, large particles ($>10\text{ }\mu\text{m}$) will be filtered out of the inhaled airstream by the aerodynamic filters of the respiratory tract, whereas smaller particles are deposited in the tracheobronchial tree or alveoli [Churg et al., 1985; Clarke and Pavia, 1991]. Fibershaped particles have dissimilar deposition patterns related to their specific length-diameter characteristics [Lippmann, 1988]. In the upper airways, dust particles are cleared by mechanisms of sneeze and cough, or by the mucocilliar escalator, and ultimately be swallowed [Clarke and Pavia, 1991; Bohning and Lippmann, 1992]. In the alveolar region, the majority of the particles are ingested by phagocytotic cells such as macrophages and neutrophils. Particles may be cleared by translocation via the alveolar epithelium upstream for mucocilliar clearance, or to the interstitium for clearance by interstitial macrophages via the regional lymph nodes [Bohning and Lippmann, 1992].

Intrinsic (chemical, morphological) properties of the dust denominate its durability and the rate to which a particle is dissolved or degraded in the lungs. Durability can range from days (e.g. cement dust) to decades (e.g. asbestos). If a particle is degradable, the alveolar macrophage will resume its normal function. However, if particles are non-degradable, the alveolar macrophage is either removed via clearance on the epithelial surface or interstitium, or remains in situ with the particle. If the diameter, or in the case of fibershaped material, the length of a particle is very large, "frustrated phagocytosis" may occur characterized by an incomplete phagocytosis [Mossman et al., 1991].

A state of "particle overload" may occur if more particles enter the lung than can be cleared from it due to impairment of macrophage clearance [Bolton, 1983; Morrow, 1992]. Thus, relatively harmless dusts can cause damage of the alveolar epithelium, and particles may reach the interstitium [Driscoll et al., 1990]. Ultrafine particles are considered to cross the epithelial barrier into the interstitium more readily [Oberdörster et al., 1994] and noteworthy, a relative low weight-dose of ultrafine particles may comprise a tremendous numerical dose. The cumulative inhaled dose is of major importance in (chronic) effects of any particulate matter. Although the hazard of different particles may vary considerably, at sufficient high dust concentrations, all dusts are considered capable of producing irreversible lung changes [Gross, 1967].

Already in ancient time it was realized that mining and stone cutting could cause adverse health effects, and it was literally considered as slave labour. In 1556 the book "De Re Metallica" written by Agricola (1494-1555) became published. This book included an advice on how to improve working conditions of miners. In 1806, Laennec recognized a disease in the lungs of coal miners which he described as melanosis [Cotes et al., 1987]. Nowadays it is known that mineral dusts may cause a variety of lung disorders. In Table 1.1, some examples of mineral dusts and related diseases are listed. It should be stressed that the causality of some of the disorders as forwarded from several studies is still subject to debate, and regularly rejected by several investigators. For instance, it is

accepted that asbestos fibers may cause bronchogenic carcinoma or mesothelioma, a malignant disease of the pleura [Mossman et al., 1989], while silica currently is classified as a "suspect" carcinogen by the International Agency for Research on Cancer [IARC, 1987], and was recently forwarded as a carcinogen to humans by the Dutch expert committee [DECOS, 1996]. Additionally, gastrointestinal malignancies have occasionally been observed in mortality studies in mineral dust exposed cohorts, and are associated with the mucociliary clearance route of inhaled particles [Meyer et al., 1980]. Regarding obstructive lung diseases observed in subjects chronically exposed to mineral dust, it is now realized that confounding effects of smoking should be seriously taken into account [Oxman et al., 1993; Weill, 1993].

Table 1.1 Examples of mineral dusts and disorders associated with their inhalation

Dust	Pneumoconiotic	Nonpneumoconiotic	Malignant disease
Asbestos	Asbestosis [Cooke, 1927] PMF [Mossman et al., 1990]	Lung function loss [Zedda et al., 1973]	Lung cancer [Mossman et al., 1990] Mesothelioma [Wagner et al., 1960]
Erionite/ zeolite			Mesothelioma [Baris et al., 1988]
Coal dust	CWP [Collis and Gilchrist, 1928] PMF [Cochrane, 1973]	Lung function loss [Love and Miller, 1982] Bronchitis [Rogan et al., 1973] Emphysema [Cummins, 1936]	Lung cancer [IARC, 1987] Gastric cancer [Stocks, 1965]
Kaolin	Kaolinitis / PMF [Wells et al., 1985]	Lung function loss [Altekruse et al., 1984]	
Silica	Silicosis / PMF [Silicosis and Silicate disease Committee, 1988] Acute silicosis [McDonald et al., 1930]	Lung function loss [Malmberg et al., 1993] Bronchitis [Sluys-Cremer et al., 1967]	Lung cancer [IARC, 1987]
Talc	Talcosis [Vallyathan et al., 1981]	Lung function loss [Malmberg et al., 1993] Bronchitis [Kleinfeld et al., 1992]	Lung cancer [Kleinfeld et al., 1992]

PMF: progressive massive fibrosis; CWP : coal workers pneumoconiosis

Inherent to its definition, pneumoconiosis is considered as a common feature of chronic inhalation of any dust. Current definition of pneumoconiosis is "the accumulation of dust in the lungs and the tissue reactions to its presence" [Gross, 1983]. The word pneumoconiosis was introduced in 1867 by the German pathologist Zenker, based on the Greek words for lung and dust [Meijers, 1991]. Pneumoconiosis caused by inhalation of mineral dusts have been described in various occupational settings such as granite, ceramic and pottery industries, mining and grinding of coal, asbestos, kaolin, gold, tin, and sandblasting [Cotes et al., 1987; Cullen et al., 1990]. Knowledge of the individual's (occupational) exposure history is a prerequisite to clinical examination of pneumoconiosis. A subject with radiological indication of pneumoconiosis is considered to have coal workers pneumoconiosis (CWP), in the knowledge that he has been exposed to coal dust. Similarly, in subjects chronically exposed to free silica, silicosis may develop, and asbestos exposure may lead to asbestosis. Examples of other minerals or metals that can cause pneumoconiosis are talc (talcosis), iron (siderosis), tin (stannosis), mica, tungsten, barite (baritosis), kaolin (kaolinosis), attapulgite, wollastonite and sepiolite [Rom, 1992a; Cotes et al., 1987] (see Table 1.1). Mixed pneumoconiosis may develop in subjects with chronic exposure to more than one type of dust.

In spite of their common definition, the different pneumoconioses usually vary considerably in pathologic features. On chest radiographs of CWP and silicosis, lesions are visible throughout the lung, albeit particularly (and at earlier stage) in the upper lung zones. Asbestosis is characterized by lesions in the lower lobes of the lung, and in severe cases lesions are seen in subpleural tissue. Furthermore, chest radiographs of CWP and silicosis are more often characterized by rounded opacities, while asbestos mainly gives rise to irregular shaped opacities [Merchant and Schwartz, 1992]. Lung biopsy studies have yielded marked differences in structural features of the simple coal dust lesion (i.e. macule) and the silicotic nodule, especially with regard to connective tissue content [Heppleston, 1992]. The replacement of normal pulmonary parenchyme by a connective matrix is an irreversible process, and this fibrogenic accumulation is characteristic to fibrosis [Snyder, 1988; Murray and Laurent, 1988]. Compared to silicosis, fibrogenesis is relatively mild process in early stage coal workers pneumoconiosis. Other dusts (e.g. barite and titanium) are associated with a minimal or lacking fibrotic response, and usually cause reversible types of pneumoconiosis.

Pneumoconioses are generally divided into two major categories, i.e. simple pneumoconiosis and progressive massive fibrosis (PMF). In simple pneumoconiosis discrete small lesions are scattered throughout the lung in varying profusion and with varying degrees of fibrosis. In early stages of simple fibrosis physiological lung functions are usually not reduced in CWP or silicosis, in contrast to asbestosis. PMF is characterized by development of large lesions,

with fibrosis and collapse of lung parenchyma. Very often the lesions may involve an entire lung lobe, which is reduced to a scar, with varying amounts of dust and calcification. In silicosis and CWP these severe lesions usually develop in the upper lobe, or there may be more than one discrete lesion. In asbestosis with PMF the lower lobes are more frequently involved. Lesions of PMF almost exclusively occur on a background of simple pneumoconiosis [Hurley et al., 1987; Snyder, 1988; Merchant and Schwartz, 1992].

It is now evident that chronic inhalation of mineral dusts may lead to a variety of disorders of the respiratory tract, and can reduce the quality or even quantity of life in those who have become diseased [Enterline, 1964; Cochrane 1973; Miller and Jacobson, 1985; IARC, 1987; Mossman et al., 1990; Dutch Health Council 1992; Oxman et al., 1993]. At present, mineral dust induced lung disorders are among the most widespread occupational lung diseases. Silicosis is considered the most prevalent chronic occupational disease in the world [Cullen et al., 1990]. As an example, in China 10 million people are thought to be exposed to mixed dusts, and in 1987 more than 600,000 pneumoconiosis patients were registered [Sjöstrand, 1989]. According to the NIOSH, occupational lung diseases, including pneumoconiosis and lung cancer, are considered as the leading work-related diseases, based on frequency of the problems, severity of disease and preventability [Rom, 1992b]. In 1989 in the United Kingdom, the SWORD project started, a reporting scheme for work related and occupational lung diseases formed by members of the British Thoracic Society [Seaton, 1991]. Major findings were that long latency disease risk was particularly high in construction workers, miners, shipyard and dock workers [Meredith and McDonald, 1994], and of all cases reported in 1989 ($n=2101$), 13% were pneumoconiosis [Seaton, 1991]. In mining industry, in spite of tremendous efforts to reduce exposure, pneumoconiosis has not yet ceased to exist [Cullen et al., 1990]. In the countries united in the (former) European committee for Steel and Coal (ECSC) coal dust induced lung disorders increasingly become a post-occupational problem [Soutar et al., 1986; Gautrin et al., 1994]. Mineral dust induced lung disorders are also regularly being reported in relation with other occupational exposures [Landrigan, 1987; Cullen et al., 1990; Nemery et al., 1992].

1.2 Biological markers

These facts (further) necessitate primary prevention (dust prevention), medical screening of exposed subjects, monitoring of exposure and post-exposure surveillance. Continued biomedical research on mineral dust induced lung disorders has allowed a better understanding of pathogenic mechanisms. Through these new insights tools may be generated to identify those at increased

health risk, and for medical care in those who have become diseased. Exactly these insights are considered as the hallmarks of molecular epidemiology, i.e. the incorporation of biomarkers in the field of "traditional" (occupational) epidemiology [Schulte, 1993]. Where *epidemiology* can be defined as "the study of the distribution and determinants of health-related states and events in populations and the application of the results in this study to control health problems", *molecular epidemiology* may be defined as "the incorporation of measurements of physiologic, cellular, subcellular and molecular events into epidemiologic research" [Schulte, 1993]. These measurements are also referred to as *biological markers* or *biomarkers*. Incorporation of biomarkers in epidemiologic research may reduce misclassification of exposure or resultant disease. And, perhaps most powerful, is its ability to account for variability and effect modification, i.e. the quantification of inter-individual susceptibility differences [Schulte, 1993]. In other words: biomarkers may explain why at similar exposure some get a disease and some not. A biomarker may represent any event in the continuum of events between causal exposure (E) and resultant disease (CD) (see Figure 1.1) [Perera and Weinstein, 1982; Schulte, 1989].

Within this "exposure-disease-continuum", biomarkers may be classified as markers of exposure, markers of effect and markers of susceptibility (see Figure 1.1). Exposure markers first became useful tools in "biological monitoring", especially in occupational health surveillance [Grandjean et al, 1994]. This is underscored, considering their overlap in their definitions: "Biological monitoring is the measurement and assessment of (workplace) agents or their metabolites either in tissue, secret, excreta, or any combination of these to evaluate exposure and health risk compared to an appropriate reference" [Zielhuis and Henderson, 1986]. The further one moves downwards in the framework (see Figure 1.1), the better the exposure markers improve exposure characterization: traditional (occupational) epidemiology estimates usually can be viewed as (categories of) presumed exposure, while biological markers of exposure measured at the target site, are more likely to be considered as true exposure [Schulte, 1991]. In occupational or environmental settings, exposure markers may be useful in disease prevention by generation of limit values [Grandjean et al., 1994]. An effect marker is a "measurable biochemical, physiologic, or other alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease" [Schulte, 1991]. Ideal effect markers are "signals" appearing very early in the exposure-disease continuum, i.e. closer to the time of exposure (see Figure 1.1) [Schulte, 1991, Borm, 1994]. A susceptibility marker is an indicator of an inherent or acquired limitation of an organism's ability to respond to the challenge of exposure to a specific xenobiotic substance [National Research Council, 1987]. In a biological framework of disease pathogenesis, factors determining susceptibility modify the effects of the agent of concern [Mauderly

and Samet, 1991]. Candidate biomarkers may be generated from *in vitro* studies, animal studies or studies in humans. A major advantage of animal experiments compared to human studies is the restriction of host and ambient factors [Schulte, 1991]. Human approaches include study designs familiar to epidemiologists, including cross-sectional (case-control), prospective and retrospective cohort studies and the intervention study [Schulte et al., 1993].

Figure 1.1 Framework of continuum of events in molecular epidemiology

Events between the continuum between causal exposure (E) and resultant disease (CD) can be categorized into (1) the internal dose (ID), i.e. the amount of a xenobiotic or its metabolites found in a biological medium; (2) the effective dose (ED), i.e. the amount of the xenobiotic or its metabolite that interacts with a critical subcellular, cellular or tissue target; (3) the early biological effect (EBE), representing an event correlated with, and possibly predictive of health impairment; and (4) altered structure or function (ASF) which are considered as precursor biological changes that are closely related to disease development [Perera and Weinstein, 1982; Schulte, 1989; Schulte, 1993; Borm 1994]. The resultant disease may be represented by markers that show the presence of current clinical disease (CD), as well as by markers of prognostic significance (PS) to show future development of the disease [Schulte, 1993].

1.3 Aim of the thesis

In occupational medicine, there is an increased need to focus on disease prevention, as well as to develop new approaches to study and identify susceptible populations [Cullen et al., 1990; Schulte 1991; Rall and Fouts, 1992; Weill, 1993]. In the past, traditional occupational epidemiology studies have elucidated many health risks [Schulte, 1993], including lung disorders related to high exposure to mineral dusts. Since the time that workplace exposure limits have been introduced, measurements of "physiologic, cellular, subcellular and

molecular events" in occupational settings have become more important, fortunately by newly developed and improved laboratory methods [Schulte et al., 1993]. Candidate biological markers have often been studied in cross-sectional occupational studies. Unfortunately however, follow-up of these study groups is rare, and thus the extent to which biomarkers predict disease or indicate prognosis often has not been evaluated.

The leitmotiv of this thesis is the recruitment of three groups of coal workers, previously involved in cross-sectional studies [Borm et al., 1988; Engelen et al., 1990; Janssen et al., 1992a]. Primary aim of this "follow-up" is to validate blood parameters measured in these studies as biomarkers by relating them (1) to (cumulative) coal dust exposure, (2) to the presence of coal dust induced disorders, and (3) -prospectively- to development or progression of these disorders. Furthermore, the same measurements were performed again at the end of the follow-up period (4) to analyse reproducibility and consistency of the original cross-sectional data, (5) to investigate the interrelationships between the parameters measured, and (6) to evaluate changes in the blood values in relation to longitudinal changes in disease status or (chronic) exposure. Measurement of new parameters was implemented at the end of the follow-up period (7) to forward new candidate biomarkers by cross-sectional data analysis as well as retrospective analysis of the study group.

In view of this thesis' subject, coal workers should be regarded as a representative population to evaluate biological markers within a general framework of mineral dust exposure and resultant lung disorders, since (1) -as stated before- many mineral dusts share several common disease endpoints including fibrogenesis (pneumoconiosis, PMF) and obstructive lung disease [Parkes, 1982; Rom et al., 1987; Becklake, 1992; Oxman et al., 1993], and (2) *in vitro* as well as *in vivo* experiments have yielded considerable similarities in the nature of biological responses to mineral dusts. Therefore, in addition to the generation of a set of (new) biomarkers in mineral dust induced lung disorders, this thesis aims on elucidation of new insights in biological mechanisms of mineral dust induced lung disorders in humans.

In the following chapters, the "classical epidemiology" of coal dust induced lung disorders (chapter 2) as well as their underlying biological mechanisms (chapter 3) are outlined to explain the particular choice of the markers measured in the studies described in this thesis. A short description of the study population, the study design, specific research questions and related selection of the markers are outlined in chapter 4.

COAL DUST INDUCED LUNG DISORDERS: EPIDEMIOLOGY

2.1 Historical background

Among the various naturally occurring minerals, coal has been recognized as fuel since the Middle Ages, and during the industrial revolution it became the major energy source. Originally, coal was extracted from outcrops and shallow drift mines [Cotes et al., 1987]. Technical inventions such as mechanical pumps, conveyors, and the pneumatic "jackhammer" allowed deep shaft mining and resulted in an excessive increase in production. Incidence and prevalence of "black lungs" quickly rised, also as a consequence of increased (mechanical) dust generation at the workplace [Cotes et al., 1987; Attfield and Wagner 1992]. Gradually it became realized coal workers comprised a major occupational population at risk for a variety of lung diseases [Fletcher, 1948] including Coal workers pneumoconiosis (CWP), bronchitis and emphysema [Attfield and Wagner, 1992; Dutch Health Council, 1992]. Workplace dust measurements as well as medical examination of employees were introduced to control health risks in coal workers. Standards for airborne dust were introduced to ensure working life cumulative exposure limits [Jacobsen et al., 1970].

In the 1980s, the world-wide production of coal was roughly estimated at 3 billion tons per year. At that time the coal mines in the Netherlands had already been closed for many years. Competition with other mining countries and the availability of new energy sources (natural gas) moved the Dutch Government (in 1965) to close all coal mines. Gradually coal mining became of a lesser importance for many members of the European Union, including Germany, the UK, and France. The last Belgian coal mine, situated at Zolder, recently (1992) closed. In sharp contrast, "developing" countries such as China and former Eastern bloc countries remain dependent on coal, but also in the USA coal will remain a major source of energy for considerable time as well owing to increased scarcity and cost of petroleum [Attfield and Wagner, 1992; Bowden, 1994].

In Western Europe, incidence of CWP and silicosis has increased from the beginning of this century until the fifties [Parkes, 1982]. Since that time a steady decrease in number of subjects with silicosis or CWP is observed, which has been attributed to a reduction in number of exposed subjects, improvements in occupational surveillance and to reduced exposure levels and duration [Parkes, 1982; Cullen et al., 1990]. However, from epidemiological studies in retired workers, as well as from morbidity and mortality data in the general population, a rising trend in incidence and prevalence of CWP is now observed in retired workers [Soutar and Hurley, 1986; François et al., 1988; Mahieu, 1990]. Bearing in mind the long latency period for mineral dust induced lung disease, entitlement to earlier and improved retirement benefits in general, as well as increased

possibilities for a mildly diseased miner to quit or change his job (causing a "healthy worker effect"), both are likely explanations for these observations [Attfield and Wagner, 1992]. Exposure reduction measures may also have caused some drift in the latency period of coal dust induced lung disorders. Evidently in the case of Western Europe, the drastic reduction in mining companies has played a dominant role. Epidemiological studies in the UK demonstrated a higher prevalence of CWP in retired miners versus active workers [Soutar and Hurley, 1986; Soutar 1987]. At the end of 1986 in France, a total of 40,177 men was on the compensation register, while only 3% of them were still active [Gautrin et al., 1994]. In Belgium 33,325 coal miners received compensation in the year 1991, while at this time most of the mines were already closed. These changes are of major concern in view of the knowledge that -although some handbooks previously stated otherwise- progression of coal workers disease may continue in spite of retirement, i.e. after the cessation of occupational exposure [Shennan et al., 1981; MacLaren and Soutar, 1985]. In France, among the first countries that introduced routine post-occupational surveillance (in 1980 [Gautrin et al., 1994]), "black lungs" have been designated as post-retirement disease for a considerable time [Mahieu, 1990]. While the situation has improved in Western Countries, it is should be realized that due to rapid "industrial revolutions", developing countries are now likely to be confronted with the major occupational health problems that Western countries had to cope with decades ago. Epidemiological studies in China, Brazil, Chili, Columbia, India, Korea and Zimbabwe, recently underscored that coal dust induced lung disorders should be regarded as an important health issue at present and in the future in these countries [Van Sprundel, 1990]. Besides, it is should be realized that health risks from coal dust exposure are not strictly limited to underground mining activities, but also occur in surface mining, processing of coal, and exposure to other carbon compounds including graphite and carbon black [Hanoa, 1983].

2.2 Coal dust

Although coal mining activities may result in exposure to many toxic compounds, such as nitrogen oxides, carbon dioxide, radon and other gases, respiratory hazards in coal miners are considered to be almost exclusively a consequence of exposure to dust that has become airborne during extraction or processing of coal [Cotes et al., 1987]. The predominant component of coal is carbon, formed by decomposition and compression of forest trees and related vegetation during the Carboniferous period, some 250 million years ago. Apart from its occurrence as coal, elemental carbon may appear in other forms such as graphite (i.e. crystalline carbon), carbon fibers and carbon black which is a product of partial oxidation or thermal decomposition of hydrocarbons [Cotes et al., 1987].

Carbon or graphite fibers are filamentary forms of carbon produced by high temperature processing of organic precursors as rayon, (coal tar) pitch or polyacrylonitrile [Vu, 1994].

Coal extraction exposes miners to a variety of minerals from surrounding layers which may become airborne during extraction of the coal, as well as to other mineral components of the coal dust particles that have become intermingled with the carbon due to earth crust movements. Coal dust may contain quartz, clay, shale, limestone form skeletons and shells of aquatic animals, and other chemical compounds such as iron and calcium. In Europe, the mineral fraction of coal mine dust (i.e. ash content) contains generally 5 to 50% mica, 4 to 30% kaolin and 2 to 20% quartz [Préat, 1993]. Traditionally, coal dust induced lung disorders have been studied in relation to specific composition of dust generated during specific jobs or in specific workplace environment. As an example, exposure to (free) silica may be relatively higher in stone roadways compared to the dust at the coal faces where coal is extracted. While high exposure to free silica may result in silicosis, the relation between quartz content in coal dust and CWP remains obscure, since both positive and negative associations with quartz content and pneumoconiotic risk have been reported [Préat, 1993]. Adverse effects of coal dust are also related to coal rank, which denominates the purity i.e. the quality of the coal as energy source. The high prevalence of CWP associated with high rank anthracite (92-94% pure carbon) compared to bituminous coal (<92%) has been attributed to the higher amount of respirable dust generated during mining, as well as to the slower clearance rate from the lung of the former [Cotes et al., 1987]. Thus, many of the described dust properties have been considered to explain for differences in disease prevalence and incidence among mining regions [Gautrin et al., 1994] or among horizontals, or specific jobs within a single mine [Reisner and Robock, 1976]. In this regard, differences in coal extraction and processing techniques among various regions or countries should not be neglected [Préat, 1993].

2.3 Coal workers pneumoconiosis

The major respiratory health effect caused by chronic coal dust exposure is CWP, characterized by a relative mild interstitial inflammation and fibrosis at early stage disease. Although pneumoconiosis in coal workers originally was attributed to silica only, gradually it became accepted that coal played an important role. In 1928, Collis and Gilchrist demonstrated that pneumoconiosis among coal trimmers arose from exposure to coal dust and not only from silica exposure [Collis and Gilchrist, 1928]. In spite of its name, CWP is not strictly limited to coal workers, since it may occur in workers exposed to any carbon dust: Exposure to graphite has been shown to result in disease almost

indistinguishable from CWP [Gaensler et al., 1966; Okutani et al., 1963], and progressive massive fibrosis (PMF) has been reported in carbon electrode workers [Watson et al., 1959]. Resembling disease has also been observed in oil shale workers [Seaton et al., 1981a]. In CWP, pathology is limited to the lower part of the respiratory tract, and primarily the respiratory bronchioles and alveolar walls are involved [Rom and Crystal, 1991]. Simple CWP is characterized by the formation of macules (or maculae), which are foci of macrophages loaded with dust accumulated in and around the respiratory bronchioles [Cotes et al., 1987, Flint, 1988]. These macules are scattered over the entire lung, but predominantly in the upper lobuli of the lung [Flint, 1988]. At more pronounced disease stage, macules become increased in size, and are also found in the pulmonary arterioles and venes. In the regions of the respiratory bronchioles reticuline and collagen becomes deposited [Flint, 1988; Snyder, 1988].

Development of progressive massive fibrosis (PMF) is due to progressive enlargement of a single coal macule, or by enlargement and fusion of adjacent nodular lesions [Cotes et al., 1987]. A typical PMF biopsy lesion appears as a black mass from which nearly all structural features have disappeared. The mass often engulfs bronchi and pulmonary arteries and extends across septa or from one lobe to another. In PMF, occasionally hypertrophy of the right ventricle and evidence in the body of right ventricular overload is observed [Cotes, 1987]. Sometimes CWP is accompanied with rheumatoid arthritis, known as Caplan's Syndrome [Cotes et al., 1987; Flint, 1988]. Ultimately, death may result from insufficient gas exchange, right-sided heart failure, or overwhelming tuberculosis or infections [Cotes et al., 1987].

A reduced life expectancy of coal workers with progressive massive fibrosis (PMF) was already reported several decades ago [Cochrane 1973]. Support for an increased mortality in simple pneumoconiosis as well as in the absence of CWP was provided by Miller and Jacobson, who additionally showed a correlation with mortality and exposure [Miller and Jacobson, 1985]. Several studies have focussed on the relation between (cumulative) exposure and pneumoconiotic lesions. Exposure assessments have been included in some of the epidemiological surveys in miners allowing to study the concept of a exposure response relationship in coal dust induced lung disorders. In spite of some limitations (e.g. dust measurement bias), these studies have lead to an indication of a general relationship between mass concentration of respirable dust and the incidence of CWP [Cotes et al., 1987], and have been extrapolated to dust control standards (usually in a range of 2 to 4 mg/m³) [Prat, 1993]. Other studies included dust measurements, as well as specific exposure characterization (e.g. coal rank, quartz content, frequencies of high peak-exposures) to explain for regional prevalence differences [Gautrin et al., 1994] development of PMF [Hurley et al., 1987], or rapid developing silicosis [Seaton et al., 1981b].

2.4 Chest radiography examination of pneumoconiosis

Traditionally, clinical diagnosis of CWP (and other pneumoconioses) is predominantly based on chest radiography. Symptoms as cough and pleghm (i.e. dark sputum) are generally considered as nonspecific symptoms of CWP, since these are also observed in coal dust related chronic bronchitis [Davis and Calhoun, 1988]. As it was realized that this should be optimally standardized, the International Labour Office (ILO) has provided a (regularly revised) classification protocol for chest radiographs. The most recent version dates from 1980 [Parmeggiani, 1983]. It is applied for medical surveillance, in clinical evaluation, and (mainly) in epidemiologic studies. The classification is based on number and size of opacities, and, as indicated before, should be regarded together with a good occupational history to avoid classification error. Other diseases such as bronchiolar carcinoma or sarcoidosis may mimic the rounded opacities of pneumoconiosis [Merchant and Schwartz, 1992]. Based on the number of small opacities in the lung three categories exist i.e. 1, 2 and 3; absence of opacities is classified in category 0. Each of these categories can be further subdivided into three subcategories, resulting in a total of 12 categories: -/0, 0/0, 0/1, 1/0, 1/1, 1/2, 2/1, 2/2, 2/3, 3/2, 3/3, 3/+. Each chest radiograph is classified into a main category, which is followed by the classification which is also considered. Size and shape are classified as p, q, or r (round) or s, t, or u (irregular), based on their size being smaller than 1.5 mm, between 1.2 and 3 mm, or between 3 and 10 mm in diameter respectively. Irregularly shaped opacities are mainly observed in asbestosis, while rounded opacities are merely characteristic for CWP or silicosis. A chest radiograph is classified into progressive massive fibrosis (PMF) if the size of the opacities exceed a diameter of 10 mm. If the diameter of a single lesion (or the sum of diameters of multiple lesions) ranges from 1 to 5 cm, the radiograph should be classified as A. Lesions with a diameter exceeding 5 cm, which have a total area smaller than 1/3 of the right lung are classified as B. If the area of the lesions exceed this size, a chest radiograph is denominated into category C.

Limitations of classification of pneumoconiosis have been continuously discussed for a considerable time [Epler et al., 1978]. Major problems in the classification are (1) the assessment of progression of pneumoconiosis, and (2) inter- and intra-reader variability. The latter appears to be especially high at low profusion scores [Begin et al., 1991]. Computed tomography has been applied recently in the clinical evaluation of pneumoconioses, giving rise to increased sensitivity in radiological identification at early stages [Bégin et al., 1991; Bégin et al., 1993; Remi-Jardin et al., 1990; Lamers et al., 1994]. Determination of progression from chest radiographs can be obtained by independent interpretation or, more preferably, by side-by-side readings [Liddell, 1978].

2.5 Nonpneumoconiotic effects of coal dust

In addition to the development of pneumoconiosis several other lung diseases have been attributed to chronic inhalation of coal (and other) dusts. Most of these findings are provided by occupational epidemiology, or by biopsy/necropsy analysis of lungs of exposed individuals. Major pitfalls in epidemiological studies among coal workers are the attribution of smoking to respiratory effects, the healthy worker effect, and the loss to follow-up of miners quitting their job due to retirement or due to disease.

While evaluation of pneumoconiosis is a matter of radiographic diagnosis, nonpneumoconiotic effects are studied by means of a variety of methods, including questionnaires, sputum analysis, spirometry, impedance measurements, and diffusion capacity measurements [Cotes et al., 1987; Wouters et al., 1994; Jorna et al., 1994a]. Lung function measurements have been introduced in routine medical screening of coal workers many decades ago, and many lung function data are now available for epidemiological studies. Airway obstruction in spirometry is determined by FEV₁, the amount of air (Liter) that after maximal inspiration can be exhaled in one second. This is expressed in liters or as percentage of the predicted value, obtained by extrapolation from standard measurements [Quanjer, 1983; ATS, 1987]. By inclusion of measurement of the forced vital capacity (FVC), i.e. the maximal exhalable amount of air after maximal inspiration, also the FEV₁/FVC ratio (i.e. Tiffeneau value) can be determined. Lung cancer is also diagnosable by chest radiograph, but classification error may occur in the presence pneumoconiosis lesions. Questionnaires that are used in epidemiologic studies as well as in surveillance and clinical screening, may include questions on cough, shortness of breath, wheezing, pleghm, asthmatic attacks, as well as on past and current smoke habits [Becklake, 1992]. High resolution computed tomography has been implemented in the diagnosis of emphysema [Becklake et al., 1992; Wouters et al., 1994]. As mentioned before, occupational history is important in the case of pneumoconiotic abnormalities, however it should also be stressed that exposure estimates are important in nonpneumoconiotic symptoms.

Inverse relations between *lung function* and cumulative exposure have been found in several epidemiological surveys in coal miners as well as in other occupations [Kaufmann et al., 1982]. In a cross-sectional survey in British coal workers in 1973 an inverse relation between cumulative respirable dust exposure and FEV₁ was found, independent of the presence of CWP [Rogan et al., 1973]. This relation was later confirmed in longitudinal surveys in coal workers in the UK [Love and Miller, 1982] and in the USA [Attfield, 1985], and also showed the attribution of age, length and smoking on FEV₁ decline [Marine et al., 1988; Attfield and Hodous, 1992]. Support for the hypothesis that lung function decline due to coal dust exposure may be underestimated due to the healthy worker

effect was provided by Soutar and Hurley. By inclusion of retired miners in the study cohort, they found that lung function loss was more severe in these subjects, as well as in those who voluntarily changed their job, compared to active workers [Soutar and Hurley, 1986]. However, despite this evidence there is still debate as to how far coal dust exposure can lead to severe obstruction in miners without CWP considering the confounding role of smoking [Lapp et al., 1994].

In 1895 Osler concluded that CWP is associated with *chronic bronchitis* [Wouters et al, 1994]. Chronic bronchitis is a morphologically defined disease, which may be caused by irritation by gases and/or deposition of particulate material on the airways, resulting in hyperplasia of mucus-producing cells lining the airways and an increase in the size and secretory activity of the mucous glands in the walls of the bronchi [Abraham, 1992]. The significant attribution of dust exposure in the prevalence of *chronic bronchitis* was reported in British coal workers [Rae et al., 1970; Rogan et al., 1973], as well as in non-smoking bituminous coal workers in the USA [Kibelstis et al., 1973]. Soutar and Hurley [1986] found increased bronchitis symptoms (chronic cough, pleghm) in retired workers compared to active workers.

Emphysema is defined by pathologic criteria, as a condition of the lung characterized by abnormal, permanent enlargement of the air spaces distal to the terminal bronchiole accompanied by destruction of their walls [Fletcher and Pride, 1984; ATS 1987]. In coal workers, emphysema is believed to be a result of weakening and dilatation of the bronchiole wall caused by accumulation of dust-loaded macrophages in first- and second-order respiratory bronchioles, enmeshed by reticulin, elastin, and collagen [Morgan and Lapp, 1976; Rom, 1990]. The association between coal dust exposure and lung emphysema was already reported by Cummins in the year 1936, based on post-mortem examination of pneumoconiotic lungs [Cummins, 1936]. Later, this relationship was further underscored by others [Lyons et al., 1981; Cockcroft et al. 1982; Ruckley et al., 1984].

The role of coal dust exposure and *lung cancer* remains a controversial issue [Silicosis and silicate disease committee, 1988] as the confounding effect of smoking is especially important in this relation. While the body of evidence for asbestos as a carcinogen has been increased tremendously over the last decades, the relation between lung cancer and exposure to silica as well as (silica containing) coal dust is not confirmed [McDonald, 1989], although crystalline silica has been classified as a suspect (bronchogenic) carcinogen by a working group of the International Agency for Research on Cancer [IARC, 1987]. It was concluded that there was sufficient evidence from animal studies, but limited evidence for human carcinogenicity of silica and that confounding factors such as smoke cannot be ruled out [IARC, 1987]. An update of the IARC monogrpahs is due in september 1996. In the Netherlands, silica has been classified as human carcinogen by inhalation very recently by the Dutch expert group [DECOS, 1996].

Regarding coal dust, mortality studies in coal workers generally have not yielded increased standardized mortality ratios (SMR) for lung cancer in (former) coal workers [Miller and Jacobsen, 1985; Meijers et al., 1991]. Based on these observations, the Dutch Health Council stated that there is 'no reason to assume that labour in the mines, whether causing CWP or not, induces an increased risk for lung cancer [Dutch Health Council, 1992]. Recently, an increased risk was found with long term exposure to coal dust, in a case-control study among lung cancer patients [Morabia et al., 1992]. Rat inhalation studies have yielded both positive [Martin et al. 1975] and negative [Karagianes et al., 1981] results with regard to lung carcinogenicity. In addition to silica, also coal dust will be evaluated by a IARC working group in 1996. From epidemiological studies a relation between coal dust exposure and cancer in the gastrointestinal tract has also been suggested [Stocks, 1965, Matolo et al., 1972; Miller and Jacobsen, 1985; Meijers et al., 1991]. Support for a role of pulmonary clearance in the development of gastrointestinal malignancies [Meyer et al., 1980], was recently obtained in a mortality study in Dutch coal miners, in which the gastric cancer risk turned out to be highest in those with normal FEV₁ [Swaen et al., 1995].

COAL DUST INDUCED LUNG DISORDERS: MECHANISMS

3.1 Particles in the lung: the role of phagocytes, epithelial cells, and fibroblasts

The alveolar macrophage is considered as a key cell in mineral dust induced lung disorders [Heppleston and Styles, 1967; Lugano et al., 1984; Driscoll et al., 1990]. Alveolar macrophages are derived from bone marrow monocyte [Thomas et al., 1976] or partly renewed by proliferation of local macrophages [Bitterman et al., 1984; Spurzem et al., 1987], and have a life-span of several months to years. Dependent on their age, the size of alveolar macrophages ranges from 12 μm to 40 μm [Crystal, 1991]. The crucial role of alveolar macrophages in mineral dust induced disorders is based on several observations:

- (1) Subjects chronically exposed to mineral particles have increased number of alveolar macrophages in the lungs, and these macrophages contain ingested particles [Takemura et al., 1989].
- (2) Macrophages have the ability to ingest several inorganic particles in vitro [Takemura et al., 1989] as well as in vivo in the lung [Ziskind et al., 1976; Brody et al., 1982]. Macrophage action is considered to be relatively independent of the fibrogenic potential of the particle [Bowden, 1987].
- (3) Phagocytosis has been shown to result in an activation state of the macrophage, and during its activation a wide range of products including oxidants, bioactive lipids, cytokines, growth factors, proteases and antiproteases may be released.

Several lung cells may act as targets for these mediators including fibroblasts, epithelial cells and endothelial cells, and all these cell types are capable of releasing secondary mediators. Epithelial cells may also act as direct targets for some of the inhaled particles, resulting in (ir)reversible injury of the epithelium. With regard to mineral dust exposure related bronchitis, bronchial epithelial cells may thus be irritated. This may cause hyperplasia of mucus producing cells and glands in the walls of the bronchi leading to increased mucus production, as well as impaired function of ciliated cells resulting in impaired mucocilliary clearance [Abraham, 1992]. In addition, epithelial cells and (in the case of asbestos fibers) mesothelial cells, may act as target cells in lung carcinogenesis including bronchial carcinoma and mesothelioma [Mossman et al., 1990]. Additional inflammatory cells (e.g. neutrophils) may be recruited from the peripheral blood by factors released by alveolar macrophages as well as by other lung cells. Recruitment of neutrophils is considered to be independent of the fibrogenicity of the particle and will lead to increased inflammatory state in the alveolar region, known as alveolitis [Bowden, 1987; Driscoll et al., 1990]. Thus, in addition

to direct damage by particles, alveolar epithelial cells may be damaged by inflammatory mediators. This results in the destruction and/or remodeling of the alveolar epithelium: Oxygen uptake may be impaired due to loss of functional alveolar type I cells by proliferating type II cells or by fibroblasts resulting in alveolar fibrosis [Chrystal et al., 1991]. Degeneration of air space walls may also cause emphysema [Abraham, 1992]. Regarding pulmonary fibrosis, interstitial fibroblasts are considered as key cells [Murray and Laurent, 1988]. This may also be the case in coal (and other mineral) dust induced fibrosis, underscored by the following observations:

- (1) Lung fibroblasts are a major source of collagen and other interstitial matrix components [Hance et al., 1976].
- (2) Development of fibrosis is characterized by the replacement of normal pulmonary parenchyme with a collagen matrix [Snyder 1988, Murray and Laurent, 1988].
- (3) Activated macrophages release several factors that have been shown to stimulate fibroblast chemotaxis, growth, proliferation and/or its collagen production. This concept has been originally proposed by Heppleston and Styles [1967], based on the observation that soluble factors released by silica-damaged macrophages were able to stimulate collagen production by cultured fibroblasts.

Although many interactions have been described to date, mechanisms of mineral dust induced lung disorders can be arbitrarily subdivided into two major pathways, involving (1) the production of reactive oxygen species and (2) the release of cytokines and growth factors. Both pathways are based on the concepts of macrophage activation and inflammation, and are considered to lead to diverse respiratory effects that are observed in chronic exposure to mineral dust. Formation of reactive oxygen species may additionally be mediated by intrinsic properties of inhaled mineral dusts, i.e. by non-cellular mechanisms. These biological pathways have been applied to create an exposure disease continuum of coal dust exposure and related diseases that has been described on the previous paragraphs (see Figure 3.1). Since both reactive oxygen species and cytokines are involved in lung diseases other than pneumoconiosis, this review will not be strictly limited to pneumoconiotic pathways of coal dusts. On the other hand, many of the *in vitro* and *in vivo* studies have been carried out with mineral dusts other than coal dust, particularly silica and asbestos. Considering the similarities in the mechanisms of mineral dust toxicity, they are included.

Figure 3.1 Framework of continuum of events related to mineral dust exposure and resultant disease. Within the exposure disease continuum, several events may be distinguished: Exposure to mineral dust (e.g. coal dust) may lead to inhalation of particles. Factors that determine the amount of dust finally reaching the lower part of the respiratory tract (internal dose) are related to the dust (e.g. particle shape and size), to the environment in which the exposure takes place (e.g. years of employment, occupational hygiene), as well as to the exposed individual (e.g. nose versus mouth breathing, tidal volume). Also the effective dose is related to dust (durability) and host (e.g. cough, mucociliar clearance, macrophage clearance). Early biological effects may include altered clearance (irritation of airways), and inflammatory responses may occur resulting in altered cellular defense mechanisms (phagocytosis, neutrophil recruitment). Particle overload may cause persistent inflammation. Particles themselves (e.g. particle shape, surface radicals), as well as cell mediated formation of reactive oxygen species, and release of cytokines and other mediators may have effects on surrounding cells and tissue. This may result in (ir)reversible cell- and tissue damage or proliferation (oxidative damage, fibrogenesis), eventually leading to altered lung structure and/or function. Clinical diagnosis may include shortness of breath, sputum/pleghm, reversible airway obstruction (bronchitis), and radiologic detection of pneumoconiotic lesions (e.g. formation of dust maculae). Eventually, disease may progress into more severe disorders (e.g. chronic bronchitis, severe lung function loss, emphysema, massive fibrosis, lung cancer).

3.2 Oxygen damage and antioxidant defense

3.2.1 Reactive oxygen species and antioxidants

Reactive oxygen (ROS) species are implicated in a variety of diseases. The most important cellular effects of ROS are DNA damage, lipid peroxidation and oxidation of proteins (see paragraph 3.2.3). Reactive oxygen species include superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^{\bullet}$), as well as some "reactive" oxygen derivatives that do not contain unpaired electrons, such as hydrogen peroxide (H_2O_2), singlet oxygen (1O_2) and hypochlorous acid ($HOCl$) (see Table 3.1). In addition, also carbon-centered radicals (e.g. $CCl_3^{\bullet}$), nitrogen radicals ($NO^{\bullet}$, $NO_2^{\bullet}$), and sulfur centered thiylradicals ($RS^{\bullet}$) are believed to be involved in human disease [Halliwell and Cross, 1994; Kehler, 1993]. Exogenous compounds that are known to cause production of ROS include ozone (O_3), nitrogen oxides, bleomycin, paraquat, and radiation.

Cellular sources of ROS comprise various soluble and membrane-bound enzymes, including cytochromes P450 and b5, the mitochondrial electron transport system, and the cyclooxygenase pathway. In 1973 Babior described that activation of phagocytotic cells leads to generation of $O_2^{\bullet-}$ [Babior et al., 1973]. In the lung, primarily macrophages and neutrophils are involved in this "respiratory burst", which utilizes plasma membrane bound *NADPH oxidase*. Upon activation of this enzyme by (opsonized) particulate stimuli via phagocytosis or by soluble stimuli such as phorbol esters, electrons are shuttled from cytosolic NADPH to oxygen to produce a molecule of $O_2^{\bullet-}$. Neutrophils additionally contain large amounts of *myeloperoxidase* which converts H_2O_2 into hypochlorous acid ($HOCl$). The highly reactive $HOCl$ is readily converted to chlorine or can react with primary or secondary amines to form relatively long-lived chloramines [Klebanoff et al., 1968; Klebanoff et al., 1980]. Phagocytotic cells are also known to produce nitric oxide ($NO^{\bullet}$) radicals [Halliwell and Cross, 1994]. $NO^{\bullet}$ is formed during the oxidation of the amino-acid L-arginine. $NO^{\bullet}$ may react with oxygen to the $NO_2^{\bullet}$ radical, or with superoxide anion to peroxynitrite ($ONOO^-$) which may be directly toxic to cells, or in turn lead to the formation of $OH^{\bullet}$ and related radical compounds [Beckman et al., 1990]. *Xanthine oxidase*, which is found in peroxysomes, generates $O_2^{\bullet-}$ and H_2O_2 during the conversion of xanthine or hypoxanthine to uric acid [Schraufstatter and Cochrane, 1991].

$O_2^{\bullet-}$ dismutates enzymatically by the superoxide dismutase enzymes (see next session) or spontaneously [Fridovich, 1974]. In the latter case, in addition to H_2O_2 also the highly reactive singlet oxygen (1O_2) is formed. $O_2^{\bullet-}$ and H_2O_2 are involved in the formation of the hydroxyl radical ($OH^{\bullet}$), which is the most highly reactive oxygen radical known [Halliwell and Cross, 1994]. This redox reaction occurs in the presence of transition metal ions such as iron and copper

salts. This iron catalysed reaction is known as the Haber-Weiss reaction [Haber and Weiss, 1934]:

Mammals possess enzymatic antioxidant mechanisms as well as nonenzymatic antioxidants to cope with the toxic effects of reactive oxygen species. Enzymatic antioxidants are the superoxide dismutases (SOD), catalases and the glutathione peroxidases. Nonenzymatic antioxidants include the vitamin A precursor β -carotene, glutathione, ascorbic acid (Vitamin C), uric acid, α -tocopherol (Vitamin E), transferrin, ceruloplasmin, lactoferrin, albumin and taurine (see Table 3.1).

Table 3.1 Reactive (oxygen) species and antioxidants

Reactive (oxidant) species	Important antioxidants	Comment
$\text{O}_2^{\bullet-}$ (superoxide anion)	Superoxide dismutases (SOD) Glutathione (GSH) Vitamins A, C, and E	formation of H_2O_2 formation of $\text{GS}^\bullet$ and H_2O_2
$\text{OH}^\bullet$ (hydroxyl radical)	Vitamin C	formation of Vitamin $\text{C}^\bullet$
H_2O_2 (hydrogen peroxide)	Catalase Se-dependent glutathione peroxidase (Gpx)#	formation of H_2O formation of H_2O
$^1\text{O}_2$ (singlet oxygen)	Vitamins A, and E, uric acid	quenching
LOOH (lipidhydroperoxides)	Gpx #, Glutathione-S-transferase (GST)#	Termination of lipid peroxidation
HOCl (hypochlorous acid)	Taurine	

* indicates a free radical; # GSH is required as substrate

Enzymatic antioxidants

Eukaryotic cells contain two forms of *superoxide dismutase* (SOD). The copper-zinc containing enzyme (CuZnSOD) is found in the cytoplasm [Crapo et al., 1992], in peroxysomes [Keller et al., 1991] as well as extracellularly [Marklund 1984]. Manganese SOD (MnSOD) is present in the mitochondria of eukaryotic cells.

The superoxide dismutase enzymes convert $\text{O}_2^{\bullet-}$ radicals to hydrogen peroxide. The *catalase* enzymes are involved in the reduction of hydrogen peroxide to water. Intracellular catalase is located in peroxysomes. Extracellular

catalase is found in blood plasma [Leff et al., 1993] and epithelial lining fluid [Cantin et al., 1985].

There are two classes of *glutathione peroxidase* enzymes. The selenium dependent glutathione peroxidase (Gpx) is known to reduce hydrogen peroxide to water, during which reduced glutathione (GSH) becomes oxidized. Oxidized glutathione (GSSG) in turn is reduced to GSH in a NADPH dependent reaction catalysed by the enzyme glutathione reductase (GR). The NADPH is provided by the hexose monophosphate shunt. This glutathione redox cycle reaction occurs in the cytosole. The selenium independent glutathione peroxidase, which is also known as *glutathione-S-transferase* (GST), is not able to reduce hydrogen peroxide to water. Gpx as well as GST reduce lipid hydroperoxides into lipid alcohols, a reaction which also involves oxidation of GSH to GSSG. In addition to its scavenging role in lipid peroxidation, GST plays a role in the detoxification of electrophilic compounds in GSH conjugation reactions. GST is found in cytosole and microsomes. Human cytosolic GST can be divided into class A, M, P and T (α , μ , π and θ). Polymorphism in humans has been described for GSTM [Seidegard et al., 1986].

Nonenzymatic antioxidants

In addition to its role as cosubstrate in the glutathione redox cycle, *glutathione* may also act as a direct antioxidant. Scavenging of superoxide anion by glutathione leads to the formation of a sulfur-centered thiyl radical ($GS^\bullet$) and hydrogen peroxide. Furthermore, glutathione is known to scavenge hydrogen peroxide and other peroxides. Glutathione is found intracellularly, as well as extracellularly in the blood plasma and epithelial lining fluid [Cantin et al., 1985]. Because of its abundance in many biological systems, glutathione is believed to be one of the main detoxificants of xenobiotics. As already mentioned above, the enzyme *Glutathione-S-transferase* is involved in the conjugation of glutathione, and a reduced activity or availability of Gpx or GST may have consequences for GSH detoxification action.

Together with glutathione, *ascorbic acid* (Vitamin C) is the most important water soluble antioxidant, and is found both intracellularly and extracellularly. Vitamin C can scavenge both $O_2^{\bullet-}$ and $OH^\bullet$ and the resulting vitamin C free radical subsequently can be reduced by glutathione. On the other hand vitamin C has prooxidant action e.g. by its ability to convert Fe^{3+} to Fe^{2+} thus likely promoting Fenton-type reactions (eq. II).

Vitamin E (α -tocopherol) is the most important lipid soluble antioxidant, and limits membrane injury in human tissue. *Vitamin E* acts as a chain reaction terminator in the lipid peroxidation process, during which it becomes a radical itself, which however is fairly stable compared to the lipid radicals [Burton and Ingold, 1981]. Furthermore, Vitamin E has antioxidant action for the superoxide radical, and it is known both to quench and react with for singlet oxygen.

Other singlet oxygen quenchers include the lipid soluble *Vitamin A* and *uric acid*, which is formed by catabolism of purines. Vitamin A also can scavenge $O_2^{\bullet-}$ and peroxy radicals [Foote and Denny, 1968]. Uric acid acts as a scavenger of $OH^{\bullet}$ and peroxy radicals, it prevents oxidation of vitamin C and it binds to transition metals [Ames et al., 1981]. Other antioxidants include iron or copper chelators as transferrin [Pacht et al., 1988], ceruloplasmin [Goldstein et al., 1979] and albumin [Brown et al., 1989a; Halliwell, 1988], and the amino-acid *taurine* which detoxifies $HOCl$ [Klebanoff et al., 1980].

3.2.2 Reactive oxygen species and antioxidants in mineral dust induced lung disorders

Adverse effects of ROS in the lung may include (1) damage to cell membranes by lipid peroxidation, (2) oxidation of proteins, and (3) DNA damage. Lipid peroxidation is a chain reaction process which involves an initial reaction of a free radical with an unsaturated fatty acid of the cellular membrane resulting in the formation of a lipid radical which reacts with oxygen to form a lipidperoxy radical. This lipidperoxy radical in turn may react with another unsaturated membrane fatty acid resulting in a reactive lipidhydroperoxide molecule and a new lipid radical. The resulting chain reaction may lead to cell damage and tissue remodeling. Reactions of ROS with proteins may lead to inactivation of enzymes involved in cell metabolism or in modification of intra- or extracellular structural components. Oxidative DNA damage may have various consequences ranging from cell death and tissue destruction to cell or tissue proliferation [Janssen et al., 1993]. A role for ROS has been considered in the pathogenesis of several lung diseases, including hyperoxic injury [Chvapil et al., 1975], radiation pneumonitis [Gross et al., 1977], collagen-vascular diseases [Hunninghake et al., 1978], adult respiratory distress syndrome (ARDS), and idiopathic pulmonary fibrosis [Cantin et al., 1987b]. At present only the role of ROS in mineral dust induced lung disorders will be discussed. Basically, two mechanisms by which mineral dust exposure causes formation of ROS *in vivo* have been proposed:

- (1) Formation of ROS by intrinsic properties of the particles, i.e. non-cellular mechanisms.
- (2) Excessive formation of ROS by the oxidative burst of macrophages and neutrophils activated during particle phagocytosis and inflammation.

(1) *Non-cellular generation of ROS.* Over the last decades several mechanisms have been proposed regarding the noncellular toxicity of mineral particles, including the role of silanol groups on the surface of silica [Nash et al., 1966], surface charge properties related to the crystalline structure of silica and asbestos [Brown et al., 1989b; Fubini, 1993], and the iron content of asbestos fibers [Zalma et al., 1987]. Grinding i.e. cleavage of coal dust (as well as silica) is believed to

cause generation of radicals on "fresh" surfaces [Vallyathan et al., 1988; Dalal et al., 1989]. The iron present on the surface of asbestos fibers may be involved in the formation of hydroxyl radicals via the Fenton-reaction (eq. II) [Weitzman and Graceffa, 1984; Goodlick and Kane, 1986; Zalma et al., 1987]. Iron content may also play an important role in the toxicity of coal dust [Tourman and Kaufmann, 1994; Dalal et al., 1995]. Fenton-reaction type formation of hydroxyl radicals was found to be positively correlated to iron content of coal dust [Dalal et al., 1995].

(2) *Cell-mediated generation of ROS*. Indirect generation of radicals is considered to be controlled by the alveolar macrophage. Macrophages incubated with mineral dusts produce excessive amounts of oxygen radicals [Kamp et al., 1992], as well as chemoattractive factors for other inflammatory cells including monocytes and PMN which in turn may produce ROS and amplify local radical formation. Also physical and chemical properties of particles were found to be related to the extent to which ROS are generated from phagocytic cells [Hansen and Mossman, 1987], and very large fibershaped particles can cause extracellular burst by incomplete or "frustrated" phagocytosis by phagocytotic cells [Mossman et al., 1987a]. Further support for increased hydroxyl radical generation in the course of pneumoconiosis disease was recently obtained in an *in vivo* silica model of fibrosis [Shapira et al., 1995].

Evidence for the involvement of ROS in humans exposed to mineral dust was initially based on observations from bronchoalveolar lavage studies. Alveolar macrophages of mineral dust exposed subjects produced increased amounts of oxygen radicals compared to non-exposed subjects [Voisin et al., 1985; Rom et al., 1987]. Comparison of oxidant generating capacities of alveolar macrophages in simple CWP versus PMF yielded a possible correlation with disease severity [Wallaert et al. 1990]. Electron spin resonance demonstrated -in line with the presence of radicals on coal and silica dusts [Dalal et al., 1989]- increased amounts of radicals in lung biopsies, and more specifically in lymph nodes, of exposed subjects compared to controls [Dalal et al., 1991].

ROS are also considered to play a crucial role in the pathogenesis of emphysema by oxidative damage to antiproteases reducing its activity. Reduced antiprotease activity may cause exaggerated lung tissue destruction by proteases [Carp et al., 1982]. Similarly, it has also been suggested that dust mediated oxidant generation may be involved in the focal emphysema in coal workers [Rom, 1990]. The FeSO_4 content of coal dust (which is formed from oxidation of pyrite/ FeS_2) has also been suggested to play a dominant role in antiprotease inactivation [Huang et al., 1994].

The role of ROS in carcinogenesis has been studied extensively [Kensler and Trush, 1984; Trush and Kensler, 1991]. Oxidative mechanisms are also likely implicated in malignant effects observed in humans exposed to mineral dusts

[Mossman et al., 1989; Janssen et al., 1993]. In the DNA damage caused by ROS *in vivo*, both $O_2^{\bullet-}$ and H_2O_2 are most likely not directly involved. However, $OH^\bullet$ has been shown to induce various types of DNA damage, most likely in Fenton reaction related mechanisms [Aruoma et al., 1989]. Since H_2O_2 can easily diffuse through cell membranes [Chance et al., 1979], $OH^\bullet$ generation may occur extra- or intracellularly. Because of its reactivity $OH^\bullet$ will always cause damage at the site of its formation [Schraufsttter and Cochrane, 1991]. C8 oxidation of deoxyguanosine (dG), resulting in 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) is one of the most prevalent naturally occurring DNA lesions in response to ROS. The ratio 8-oxodG/dG has been forwarded as biomarker of oxidative stress [Kasai et al., 1986; Kuchino et al., 1987], and has been studied in relation to mineral dust exposure *in vitro* [Leanderson et al., 1988; Adachi et al., 1994; Takeuchi et al., 1994]. As collection of blood lymphocytes is considered as a relatively non-invasive method to obtain DNA, 8-oxodG/dG ratios in these surrogate cells are considered as relevant markers of oxidative DNA damage in target cells and tissue including the lung. Recently, 8-oxodG/dG was determined in peripheral blood lymphocytes of asbestos workers [Hanaoka et al., 1993]. Silica also causes formation of strand breaks *in vitro*, likely related to surface properties of these particles [Daniel et al., 1993].

Studies using radical scavengers or antioxidants have underscored the significance of ROS in lung toxicity [Goodglick and Kane, 1986; Mossman et al., 1987b; Voisin et al., 1985; Marsh and Mossman, 1991], including pulmonary fibrosis [Mossman et al., 1990]. Based on these observations, adverse oxidative effects have been suggested to an impaired balance between ROS and antioxidant defense status, and upregulation of antioxidant enzymes is considered as a marker of oxidative stress [Borm et al., 1986; Janssen et al., 1994]. For instance, hydrogen peroxide causes increases expression of catalase, MnSOD and Gpx *in vitro* [Shull et al., 1991], and both *in vitro* and *in vivo* studies showed that mineral dusts induce the expression as well as release of antioxidant enzymes including catalase and MnSOD [Janssen et al., 1992; Janssen et al., 1994]. Red blood cells contain large amounts of antioxidants and are considered to act as powerful antioxidant carriers for oxidative lung toxicity [Toth et al., 1984; van Asbeck et al., 1984]. Consequently, altered levels of red cell antioxidants (GSH, catalase, SOD) found in CWP likely reflect the significance of radical mechanisms in coal dust exposure and pneumoconiosis disease in humans [Borm et al., 1986; Engelen et al., 1988; Evelo et al., 1993]. Recently, altered antioxidant levels were found in bronchoalveolar lavage of coal workers compared to non-exposed subjects [Vallyathan et al., 1995]. The proposed role of reduced vitamin A and E status in pulmonary fibrosis may be related to their importance as free radical scavenger and chain reaction terminator in the lipid peroxidation process. A reduced nutrient intake of vitamins (A, C and E) has also been associated with increased risk of progressive lung function loss and airway obstruction [Morabia

et al., 1989; Schwartz and Weiss, 1994; Britton et al., 1995], and dietary vitamin A status has been considered as a "susceptibility" determinant in coal workers [Swartz et al., 1978]. Reduced extracellular GSH content (epithelial lining fluid) has been associated to increased lung fibroblast proliferation [Cantin et al., 1990], and a resulting therapeutic role in lung fibrosis for GSH has been proposed. Other antioxidant therapies currently in focus include administration of SOD or catalase-lipids. In line with the possible role of GST-M polymorphism in smoking related lung cancer [Seidegard et al., 1986, 1990], it has been suggested recently, that GST-M1 deficient subjects are at increased risk for asbestosis [Smith et al. 1994]. However, others have failed to show such genetic predisposition [Jakobsson et al., 1994].

3.3 Cytokines

3.3.1 Cytokines and mineral dust

Cytokines are polypeptides that are produced by and regulate the function of virtually all nucleated cells [Elias and Zitnik, 1992]. Cytokines share several common features. With a few exceptions, all cytokines are multifunctional in a wide spectrum of biological events including inflammation, metabolism, cell growth and differentiation, morphogenesis, fibrogenesis, and/or homeostasis [Elias and Zitnik, 1992]. Cytokine action may be concentration dependent, and is effectuated via binding to specific cell receptors. Cytokines may act autocrine, paracrine and/or heterocrine. Cytokine action is also dependent on the activation state and/or maturation of their target cells as well as the composition and state of degradation of the surrounding matrix [Elias and Zitnik, 1992]. Cytokines function in cascades or cytokine networks, i.e. cytokine effects are dependent on the presence or absence of other cytokines in a local microenvironment [Kelley, 1990]. In addition to cells of the immune system, the major cytokine sources in the lung are epithelial cells, endothelial cells and fibroblasts. This review will mainly focus on the mostly appreciated candidate cytokines in the two-step mechanism by macrophages and fibroblasts involved in pneumoconiosis disease. Table 3.2 shows cytokines as well as some other secretory products that are released by monocytes or macrophages upon *in vitro* stimulation with coal dust. Since an important role of the (toxic) properties of coal dust has been attributed to its silica content, relevant data obtained with silica in the same as well as other studies are also included. Furthermore, most important findings with asbestos are described.

Several candidate cytokines and other macrophage derived factors have been forwarded (Table 3.3) to control the hallmarks of pulmonary fibrosis, i.e. fibroblast activation, proliferation and production of extracellular matrix components. Many candidates have been forwarded from *in vitro* studies in cultured fibroblasts and are of limited value to the *in vivo* situation. More

Table 3.2 Factors released by monocyte/macrophage upon *in vitro* incubation with coal dust, asbestos, or silica

Cell /source	dust	factor	Remarks	References
Macrophage/murine	Silica	IL-1		Gery et al., 1981
Macrophage/murine	Silica, Asbestos	IL-1	TiO ₂ does not stimulate IL-1 release	Oghiso et al., 1987
Monocyte/human	Silica	IL-1		Schmidt et al., 1984
Monocyte/human	Coal, Silica	TNF		Borm et al., 1988
Macrophage/murine	Silica, Asbestos	TNF		Bisonette et al., 1989
Macrophage/human	Silica, Asbestos	TNF; LTB ₄		Dubois et al., 1989
Macrophage/murine	Silica, Asbestos	FN	TiO ₂ does not stimulate FN release	Davies et al., 1989
Macrophage/murine	Silica, Asbestos	TNF; LTB ₄	No elevated IL-1 release; TiO ₂ does not stimulate TNF or LTB ₄	Driscoll et al., 1990
Macrophage/human	Coal, Silica	TNF; IL-6	No elevated release of IL-1; silica does not stimulate IL-6	Gosset et al., 1991
Macrophage/murine	Coal, Silica	PGE ₂ ; TXA ₂	Released by "fresh" coal dust only; no stimulation of LTB ₄	Kuhn et al., 1992
Macrophage/human	Coal, Silica	PAF	Coal dust induced release approx. 20% compared to silica	Lapp et al., 1993
Macrophage/human	Asbestos	TNF	No elevated IL-1, IL-6, GM-CSF, PGE ₂ ; TiO ₂ does not stimulate any factor	Perkins et al., 1993
Macrophage/human	Coal, Silica	PDGF; TGF; IGF-I	TiO ₂ stimulates IGF-I, but not TGF or PDGF	Vanhée et al., 1994

IL-1 = Interleukin-1, TNF = Tumor Necrosis Factor- α , LTB₄ = Leukotriene-B₄, FN = Fibronectin, IL-6 = Interleukin-6, PGE₂ = Prostaglandin-E₂, TXA₂ = Thromboxane-A₂, PAF = Platelet Activating Factor, PDGF = Platelet Derived Growth Factor, TGF = Transforming Growth Factor- β , IGF-I = Insuline Like Growth Factor-I

36 Table 3.3 Major factors released by alveolar macrophages recognized for their stimulating or inhibitory effects on fibroblasts *in vitro*

Factor	Effect on fibroblasts	References
TNF	growth (+) collagen production (+) collagen gene expression (+)	Vilcek et al., 1987; Leibovich et al., 1987; Beutler and Cerami, 1987; Martinet et al., 1988 Elias et al., 1990
IL-1	growth (+) collagen gene expression (+)	Schmidt et al., 1982; Bitterman et al., 1986 Dayer et al., 1986; Elias et al., 1987 Goldring and Krane, 1987
TGF	growth (+) growth (-) chemoattraction (+) chemoattraction (0)	Ignotz et al., 1986; Fine and Goldstein, 1987; Wahl et al., 1989 Sporn et al., 1986; Yamauchi et al., 1988; Moses et al., 1990; Raghu et al., 1989 Postlethwaite et al., 1987 Osornio-Vargas et al., 1993
PDGF	growth (+), chemoattract (+) collagen gene expression (+) collagen production (0)	Martinet et al., 1987; Leibovich and Ross, 1976; Shaw et al., 1989 Narayanan et al., 1983 Clark et al., 1993
IGF-1	growth(+) collagen gene expression (+)	Bitterman et al., 1982; Rom et al., 1988 Goldstein et al., 1989
IFN	growth (+) growth (-) collagen production (-) collagen gene expression (0)	Elias et al., 1987 Hyde et al., 1988 Amento et al., 1985 Narayanan et al., 1989
FN	growth/chemoattraction (+)	Rennard et al., 1981
PGE	growth (-)	Elias et al., 1985

(+) stimulatory effect; (-) inhibiting effect; (0) absence of significant effect

TNF = Tumor Necrosis Factor- α , IL-1 = Interleukin-1, TGF = Transforming Growth Factor β , PDGF = Platelet Derived Growth Factor, IGF-I = Insuline Like Growth Factor-I, IFN = Interferon- γ , FN = Fibronectin, PGE = Prostaglandin-E

relevant are *in vivo* studies performed in animals, using mineral dust models of fibrosis (e.g. silica, asbestos, TiO₂), or bleomycin [Giri et al., 1980; Laurent et al., 1983; Raghov et al., 1985]. Additionally, some studies have used bronchoalveolar lavage cells (alveolar macrophages) or fluid from exposed animal or humans. In this review, mainly the role of the most relevant cytokines with regard to fibrosis will be discussed below. However also some nonpneumoconiotic effects that might be mediated by cytokines are briefly outlined.

3.3.2 Tumor Necrosis Factor- α and receptors.

Tumor necrosis factor- α (TNF) is the name given to a 17kDa protein released by lipopolysaccharide (LPS) stimulated murine macrophages, that caused hemorrhagic necrosis in experimental intradermal tumors in mice [Carswell et al., 1975]. It appeared to be the same protein as cachectin, a endogenous humoral mediator of cachexia in infections [Beutler et al., 1985; Cerami et al., 1985]. TNF is a crucial factor in endotoxin related toxic effects and septic shock can be prevented by TNF antibodies [Tracey et al., 1987]. TNF is predominantly released by macrophages and monocytes, but also other cells produce TNF such as neutrophils [Djeu et al., 1990] and lymphocytes [Sung et al., 1988]. Macrophages release higher amounts of TNF than monocytes [Martinet et al., 1988]. The human gene of TNF is situated on chromosome 6, linked to the gene encoding Tumor Necrosis Factor- β (or lymphotoxin), close to the Major Histocompatibility Complex (MHC) region [Schollmeier, 1990]. Important effects of TNF, which are mediated by TNF receptors, include induction of fibroblast growth [Vilcek et al., 1986; Martinet et al., 1988], collagenase and PGE₂ release [Dayer et al., 1985], expression of neutrophil adherence molecules [Gamble et al., 1985], enhancement of oxidant production from neutrophils [Klebanoff et al., 1986], macrophages [Warren et al., 1988], and fibroblasts [Meier et al., 1989] and induction of expression of antioxidants [Tsau et al., 1990; Wong and Goeddel., 1988]. TNF is chemotactic for neutrophils and monocytes [Ming et al., 1987]. TNF release may be enhanced by many agents or events including lipopolysaccharides (LPS), and complexes of LPS and LPS-binding protein (LBP), interferon- γ , IL-1, protein kinase-C activating agents, reactive oxygen species [Chaudhri and Clark, 1989], monocyte adherence, C5a, Vitamin D₃, granulocyte-macrophage and macrophage colony stimulating factors (GM-CSF and M-CSF), and ionizing radiation. Agents known to suppress TNF release include glucocorticosteroids [Waage et al., 1987] and prostaglandin-E₂ [Kunkel et al., 1988].

TNF release by monocytes/macrophages has been observed in response to several mineral dusts (see Table 3.2). Stimulation with coal dust particles results both in an enhanced mRNA expression as well as protein release in a dose response manner [Borm et al., 1988; Gosset et al., 1991]. Gosset and coworkers showed that in comparison to silica, coal dust had an extra effect on macrophage TNF release which was not attributable to its silica content [Gosset et al., 1991].

The first *in vivo* evidence of TNF in mineral dust induced fibrosis was indicated by increased spontaneous and stimulated release of TNF from peripheral blood monocytes of (pneumoconiotic) coal workers compared to controls [Borm et al., 1988]. Baseline as well as (dust) stimulated TNF release by monocytes of coal workers without CWP were increased compared to controls, and was even further increased in those subjects with early radiologic stage defined CWP [Borm et al., 1988]. Similarly, a higher spontaneous TNF release from alveolar macrophages of subjects with PMF compared to subjects with simple pneumoconiosis and non-exposed controls was reported [Lasalle et al., 1990]. Increased release of TNF was also found in macrophages of rats exposed to silica [Driscoll et al., 1990] and in alveolar as well as peritoneal macrophages [Mohr et al., 1991]. Crucial *in vivo* support for the role of TNF in mineral dust induced fibrosis was provided by experiments of Piguet and coworkers [Piguet et al., 1990]. In mice exposed to silica, total lung TNF mRNA was increased up to 70 days post exposure, and *in situ* hybridization elucidated increased TNF levels in silicotic nodules [Piguet et al., 1990]. Furthermore, administration of anti-TNF reduced the hydroxyproline content as a measure of fibrosis, while silica plus recombinant TNF (but not recombinant TNF only) increased fibrosis [Piguet et al., 1990]. More recently, in subjects chronically exposed to asbestos without asbestosis elevated baseline macrophage release of TNF (and IL1, IL6 and PGE2) was observed compared to controls [Perkins et al., 1993]. Changes in macrophage subpopulations (i.e. increased recruitment of monocytes) during development of bleomycin induced fibrosis are considered to be responsible for changes TNF release, as TNF release was demonstrated to be specific to a macrophage subset [Everson and Chandler, 1992]. The large individual variation in monocyte/macrophage TNF release is considered to be related to genetic polymorphism [Mölvig et al., 1988; Pociot et al., 1993], and may be closely related to polymorphism described for HLA [Wilson et al., 1992]. HLA related genetic predisposition to mineral induced fibrosis has been suggested for silicosis [Honda et al., 1988] and more recently for CWP [Rihs et al., 1994].

Two distinct transmembrane TNF receptors proteins have been cloned, with molecular weights of 55kD and 75kD respectively [Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990]. Release of both TNF receptor types from cellular membranes, has been described for various cells including neutrophils, lymphocytes and monocytes. The resulting *soluble TNF receptors* (sTNFr55 and sTNFr75) possibly constitute a powerful feedback mechanism for (systemic) TNF release *in vivo*. Soluble TNF receptors inhibit acute (LPS induced) inflammation [Ulich et al., 1993] and endotoxemia [Spinaz et al., 1992; Shapiro et al., 1993; Bemelmans et al., 1993]. Increased serum levels of sTNFr have been reported in cardiovascular disease [Latini et al., 1994], cirrhotic liver disease [Tilg et al., 1993], and have been forwarded as markers in malignant diseases [Aderka et al., 1991; Naylor et al., 1993]. Recently, increased bronchoalveolar lavage levels of soluble

TNF receptors were observed in several inflammatory lung diseases including idiopathic pulmonary fibrosis [Walker et al., 1994]. Similar to their previous experiments using TNF antibodies [Piguet et al., 1990], Piguet and coworkers recently demonstrated a preventive as well as curative role of infused TNF receptors in bleomycin or silica induced fibrosis [Piguet and Vesin, 1994].

3.3.3 Transforming Growth Factor- β

Transforming growth factor- β proteins are synthesized as large, biologically inactive precursors. At least three types have been described in human, i.e. TGF β 1, TGF β 2 and TGF β 3 [Kelley, 1993]. TGF β 1 and TGF β 2 are 25kDa and 24 kDa proteins, respectively, which share about 70% homology [Kelley, 1990]. Genes encoding TGF β proteins are located on chromosome 19, and major sources include blood platelets, alveolar macrophages [Vanhée et al., 1994], peripheral blood monocytes and neutrophils [Grotendorst et al., 1989]. TGF β is secreted in a latent form, but may be activated *in vitro* by detergents, proteolytic enzymes or brief exposure to extreme pH [Kelley, 1993]. Latent TGF β cannot bind to its receptors, and the activation of TGF β *in vivo* is considered as an important regulatory step. *In vivo* TGF β is believed to be cleaved by plasmin or cathepsin G [Lyons et al., 1988]. TGF β released by monocytes/macrophages appears to be preactivated as a consequence of proteolytic processing on the cell surface [Grotendorst et al., 1989]. Alveolar macrophages constitutively express TGF β . [Assoian et al., 1987]. TGF β action is mediated via several specific receptors [Wakefield et al., 1987]. TGF β is chemotactic for monocytes [Wahl et al., 1987], neutrophils [Reibman et al., 1991], and fibroblast [Postlethwaite et al., 1987]. TGF β has been reported to increase the production of collagen [Fine and Goldstein, 1987; Roberts et al., 1986] and fibronectin [Dean et al., 1988; Ignatz et al., 1986], as well as of protease inhibitors while synthesis of proteases is decreased [Kelley, 1993]. TGF β has been reported to produce collagen by fibroblast in the absence of proliferation [Raghu et al., 1986]. However, others have found contradictory results of TGF β action on fibroblasts (see Table 3.3), including the absence of chemotactic activity towards fibroblasts [Osornio-Vargas et al., 1993]. *In vitro* response TGF β is strongly dependent on cell type, presence of other growth factors, and the activation state of the cells before exposure to TGF β [Kelley, 1993]. Based on *in vitro* observations, it is suggested that the fibrotic action of TGF β may dependent on its concentration, i.e. a stimulatory effect on fibroblasts at low concentration and an inhibitory effect at higher concentrations [Battegay et al, 1990].

TGF β is also released *in vitro* by macrophages stimulated with silica or coal dust [Vanhée et al., 1994], and the *in vivo* action of TGF β in fibrosis has been studied in animal models using bleomycin and silica. In the bleomycin model, TGF β mRNA expression was considered as an early event that was followed by collagen production [Raghow et al., 1989; Hoyt and Lazo, 1990]. Increased TGF β is

also found in human fibrosis [Khalil et al., 1991], and is simultaneously expressed with fibronectin and type I procollagen [Broekelman et al., 1991]. Interestingly, Piguet and coworkers found increased TGF β mRNA in the bleomycin model [Piguet et al., 1989], but not in the silica model, opposite to TNF mRNA [Piguet et al., 1990]. Vanhe and coworkers showed that alveolar macrophage supernatants from simple CWP patients, but not from PMF patients, inhibited fibroblast growth in vitro, and that this "anti-fibrotic" effect could be counteracted by addition of anti-TGF β in the supernatants [Vanhe et al., 1995]. Recently it was demonstrated that TGF may cause increased oxidative damage in alveolar cells by means of depletion of antioxidant enzymes including glutathione, glutathione reductase and catalase [Arsalane et al., 1995].

3.3.4 Other cytokines relevant to mineral dust induced effects

Other relevant cytokines that will be discussed in view of possible involvement in the development or progression of mineral dust induced disorders are interleukin-1, interleukin-6 and platelet derived growth factor.

Interleukin-1 (IL-1) is also known as endogenous pyrogen, based on its ability to cause fever in response to infection [Atkins, 1960], or lymphocyte-activating factor (LAF) [Gery et al., 1972]. Interleukin-1 is a 17.5kDa protein. IL-1 α and IL-1 β proteins share 26% homology, but bind to an identical 80kDa IL-1 receptor [Sims et al., 1988]. IL-1 is predominantly produced by monocytes and macrophages [Wewers et al., 1989], and IL-1 production is reduced in smokers [Yamaguchi et al., 1989]. The IL-1 genes are located on chromosome 2. IL-1 is produced by a variety of activated cells, including monocytes and macrophages, B-lymphocytes, Natural Killer cells, neutrophils and fibroblasts [Dinarello and Savage, 1989]. Major sources of IL-1 are stimulated monocytes and macrophages, which predominantly release IL-1 β . Expression of IL-1 in these cells may be increased by endotoxin, phorbol esters and cytokines including TNF α and IL-1. Monocyte adherence also induces IL-1 release [Fuhlbrigge et al., 1987]. Like TNF, production of IL-1 may be attenuated by prostaglandin E2 and glucocorticosteroids [Aksamit and Hunninghake, 1993]. Recently an IL-1 receptor antagonist has become recognized as an important inhibitor of IL-1 related effects.

Several mineral dusts that are known to stimulate monocyte/macrophage IL-1 release, include silica [Schmidt et al., 1984] and asbestos [Hartmann et al., 1984]. Both mRNA expression as well as protein release of IL-1 is upregulated in monocytes or macrophages stimulated with asbestos [Zhang et al., 1993]. In contrast to TNF and IL-6, the increased mRNA expression of IL-1 in coal dust stimulated macrophages results not in an increased protein release [Gosset et al., 1991]. This discrepancy has been attributed to an effect of the interleukin-1 receptor antagonist released by the same cells [Vanhe et al., 1994]. Interestingly, Piguet and coworkers showed a significant reduction of silica and bleomycin induced fibrosis with interleukin-1 antagonist [Piguet et al., 1993], although IL-1

expression was not released in previous experiments [Piguet et al., 1990]. However, Driscoll and coworkers showed that, in line with TNF, IL-1 may be involved in experimental silicosis [Driscoll et al., 1990]. Recently, baseline IL-1 levels were found to be increased in macrophages of subjects chronically exposed to asbestos [Perkins et al., 1993].

Interleukin-6 (IL-6) is produced by most nucleated cells including monocytes, (alveolar) macrophages [Kotloff et al., 1990; May et al., 1989], endothelial cells, fibroblasts and B and T cells [Zitnik and Elias, 1993]. The gene encoding for IL-6 is located on chromosome 7. IL-6 molecules have different sizes ranging from 17kD to 85kD; monocytes preferentially release 24kD protein [May et al., 1988]. IL-6 is the common name finally given to a number of factors with various biological effects that proved to be mediated by the identical factor [Zitnik and Elias, 1993]. Previous names include IFN β 2, 26K, hybridoma-plasmacytoma growth factor (HPGF), B-cell differentiation factor (BCDF), hepatocyte stimulating factor (HSF) and B-cell stimulating factor-2 (BSF-2). IL-6 has a variety of effects that are mediated via specific IL-6 receptors (80kD). Most important function is regulation of B-cells and T-cells. IL-6 is the major regulator of the acute phase response and plays an important role in sepsis [Gauldie et al., 1990]. Several cytokines known to stimulate IL-6 release include IL-1, TNF, PDGF and TGF β [Ray et al., 1989]. In IL-6 response to LPS, macrophages are more potent than monocytes, while upon stimulation with IL-1 monocytes release more IL-6 compared to macrophages [Kotloff et al., 1991]. Potent inhibitors of IL-6 include TGF β , retinoids and glucocorticosteroids [Elias et al., 1991; Zitnik and Elias 1993]. Serum levels of IL-6 has been forwarded as a diagnostic/prognostic marker in cancer [Zitnik and Elias, 1993]. Genetic polymorphism has been demonstrated for IL-6 [Bowcock et al., 1988].

In the mouse silica-model IL-6 was found in most of the cells from the nodules, however not in serum [Piguet et al., 1990]. Human macrophages release IL-6 in response to coal dust but interestingly not in response to silica or TiO₂ [Gosset et al., 1991]. In addition to TNF and IL-1, macrophages of asbestos exposed subjects produce increased IL-6 [Perkins et al., 1993]. Increased IL-6 expression was found in the lungs of coal miners with CWP and PMF [Vanhée et al., 1995]. Recently it was found that IL-6 inhibits collagen degradation [Bienkowski and Gotkin, 1995].

Among all cytokines, *Platelet derived growth factor* (PDGF) may play a major role in fibrosis, as it is considered as the most potent stimulus of fibroblast proliferation [Ross et al., 1986]. PDGF is a 30kDa dimeric protein, composed of two A, chains, two B chains, or an AB heterodimer. The PDGF-A monomer gene is located on chromosome 7, PDGF-B on chromosome 22. PDGF has originally been isolated from human platelets [Antoniades et al., 1981], from which large amounts are released at sites of tissue injury [Ross et al., 1986]. Various other cells that are known to secrete PDGF include monocytes, macrophages [Shimokado et

al., 1985] and fibroblasts [Fabisiak et al., 1992]. PDGF mediated effects are mediated by two PDGF receptors, i.e a α -hemireceptor, which binds to PDGF-AA or PDGF-AB dimeric proteins and a β -hemireceptor which only can bind the BB dimer [Hart et al., 1988]. The α_2 -macroglobulin binds PDGF, and PDGF related effects are dependent on this binding [Bonner and Brody, 1995].

Macrophage release of PDGF may be induced by inorganic particles [Shapira et al., 1992], including coal dust [Vanhée et al., 1995]. PDGF release by fibroblasts is considered to follow an autocrine loop [Battegay et al., 1990]. Although a potent stimulant of fibroblast growth, PDGF has not been shown to induce collagen production by fibroblasts [Clark et al., 1993]. The anti-fibroproliferative action observed with TGF β may be closely related to the effects of PDGF. The proliferative effects observed in response to TGF β can be blocked by administration of anti-PDGF antibodies [Leof et al., 1986], and TGF β is likely involved in the downregulation of the PDGF receptor expression [Battegay et al., 1990]. Recently, Piguet and coworkers showed that PDGF (and TGF β) from blood platelets may play an important role in fibrogenesis [Piguet et al., 1994; Piguet et al., 1995].

3.3.5 Cytokine networks in mineral dust induced lung disorders

Although the action mechanisms of cytokines have been extensively studied *in vitro*, the final responses elicited by cytokines *in vivo* are dependent on the state of its surrounding tissue, including the presence of other cytokines, soluble cytokine (ant)agonists, as well as the expression of cytokine receptors of target cells [Elias and Zitnik, 1992]. This cytokine network action is considered to play a crucial role in lung disease [Kelley, 1990]. The complexity of the real-life situation in normal or diseased lung may be underscored by the following example.

In addition their stimulating role in fibroblast growth and/or collagen production (see Table 3.3), IL-1 TNF, as well as PDGF increase the synthesis of collagenases [Raghu and Kinsella, 1993]. TNF stimulates monocyte IL-1 release [Danis et al., 1989], induces the biosynthesis of TGF β and PDGF [Tracey et al., 1989], but at the same time it is a potent inhibitor of TGF β induced collagen production by downregulating the TGF β gene expression [Kähäri et al., 1990]. TGF β or TNF act synergistically with IL-1 in stimulation of fibroblast IL-6 release and collagen production [Elias et al., 1987; Elias et al., 1991]. However, in combination IL-1 and TNF are less potent in stimulating collagen production than TNF or IL-1 alone [Elias et al., 1987, 1990]. IFN- γ reduces the stimulatory effects of TNF or IL-1 [Elias and Kotloff, 1991]. Macrophage TGF β expression may be induced by PDGF [Pierce et al., 1989; Pierce, 1990], and TGF β in turn stimulates monocytes to produce TNF α , IL-1 and PDGF [Roberts and Sporn, 1990]. At the same time TGF β suppresses TNF stimulated fibroblast proliferation [Kamijo et al., 1989], and downregulates TNF expression in macrophages [Epsevik et al.,

1987]. PDGF receptors may be either upregulated by IL-1 [Lindroos et al., 1995] or downregulated by TGF β [Bonner et al., 1995].

In addition to these and many other possible interactions in the local microenvironment, it should be realized that pathogenic responses in the lung may be related to systemic factors. An important role in lung inflammation is the recruitment of cells of the immune system including "fresh" monocytes/macrophages and neutrophils. Potent factors in this recruitment include the cytokines IL-8 [Kelley, 1990], the Macrophage Inflammatory Proteins (MIP) [Wolpe et al., 1987], PDGF [Martinet et al., 1987], TGF β [Wahl, 1987], Monocyte Chemotactic Protein-1 [Smith et al., 1995], as well as some other molecules including Leukotriene B₄ [Snyderman and Goetzl, 1981]. Recruitment of immune cells, and factors released by these cells, such as reactive oxygen species, IL-4, IL-5, IL-6, IL-8, and interferons [Kelley, 1990] may play an important synergistic or antagonistic role in the fibrogenic effects of mineral dusts. Many of these factors were found to be elevated in rodent models of silicosis, including LTB₄ (and PGE₂) [Henderson et al., 1991], MIP [Driscoll et al., 1992; 1994] and IL-6 [Piguet et al., 1990]. Rat studies have indicated that recruitment of neutrophils may be closely related to the toxic effects of particles, e.g. of silica versus TiO₂ [Driscoll et al., 1990, 1995]. Although not discussed in detail, these and other cytokines may also be involved in nonpneumoconiotic effects in coal workers.

3.4 Collagen.

3.4.1 *Collagens in the lung*

The connective tissue of the lungs consists of collagen, elastic fibers and proteoglycans. At least 15 types of collagen have been described to date [Bienkowski, 1991; Prockop and Kivirikko, 1995], the lung interstitium containing collagen types I, III, V, VI and XII [Bienkowski, 1991]. Interstitial collagens are important determinants of structural and mechanical properties of the lung, which normally contains about 100 to 200 mg collagen (dry weight). Interstitial collagens of the lung are synthesized by fibroblasts [Hance et al, 1976], endothelial cells [Makarak et al., 1984], pleural mesothelial cells [Rennard et al., 1984] and alveolar epithelial cells [Sage et al., 1983]. A collagen is a triple helix of three polypeptide subunits known as α -chains, and contains high amounts of (hydroxy)proline. A major part of the α -chain subunits consists of repetitive amino-acid triplets containing a glycine followed by two random amino-acids. Collagen trimers are either homotrimers, i.e. composed of three identical subunits or heterotrimers, i.e. composed of two or three different subunits.

The most abundant collagens are type I and III which copolymerize into collagen fibrils and are normally found in a ratio of 3:1 to 6:1 in the interstitium [Madri and Furthmayer, 1980]. Collagen I represents about 60 to 70% of the lung collagen content. Collagen I and III are mainly produced by fibroblasts, but

additionally can be produced by endothelial cells, epithelial cells, and smooth muscle cells. Collagen type V is often associated on the surface of the large I/III fibers, whereas type VI forms an independent beaded network in the interstitium [Bienkowski, 1991]. Collagen V and VI, which are both produced by fibroblasts generally contain less than 5% of the lung. Collagen type IV, which represents about 5% of the lung mass is not found in the interstitium, but is an important component of the basement membrane of the lung. Other components of the basement membrane, which forms an important particle barrier to the interstitium, are laminin and proteoglycans. Several components of the extracellular matrix have chemotactic activity towards inflammatory cells and fibroblasts, such as collagen I, III and VI, laminin and fibronectin [Postlethwaite et al., 1976; Laskin et al., 1986].

3.4.2 Collagen synthesis and degradation

The normal lung is characterized by on-going processes of synthesis and degradation of collagens. Increased collagen deposition -the hallmark of fibrosis- is believed to result from an impaired balance between these processes [Snyder, 1988; Murray and Laurent 1988]. Collagens are synthesized and secreted as higher molecular weight molecules known as procollagens [Bienkowski, 1991]. Procollagens are trimers of pro α -chains, with two propeptides at each end, i.e. a C-terminal propeptide and a N-terminal propeptide. The C-terminal propeptides are registration sites for individual pro- α -chains and initiate the folding of the triple helix. During posttranslational modification of the collagen, hydroxyproline is formed by enzymatic hydroxylation of proline during its incorporation into a peptide chain. This reaction is mediated by prolylhydroxylase. Since no other known protein contains significant amounts of hydroxyproline, it is generally used as a marker for collagen, and increased lung hydroxyproline content is considered as a marker of fibrosis [Murray and Laurent, 1988]. Both propeptides, which allow the collagen to be soluble within the cell, are cleaved by specific peptidases after the procollagen molecule is secreted. After its cleavage, the N-terminal procollagen is believed to play a role in a feedback inhibition of procollagen translation process [Bienkowski, 1991]. Collagen molecules in a fibril are arranged in parallel. Lysine and hydroxyllysine residues are responsible for the intra- and intermolecular cross-links between the individual α -chains of the collagen units. Collagens bind to cell surface receptors as well as to fibronectin in the matrix. In normal lung diameter of collagens are uniform, but in fibrotic lung fibrils are often frayed and have irregular contours [Fukuda et al., 1985]. It is not known whether this is a result of degradation of normal fibrils or as a result of deranged fibrilogenesis.

The degradation of interstitial collagens is guided by collagenases that are synthesized by interstitial cells as well as by macrophages [Welgus et al., 1985; Garbisa et al., 1986; Hibbs et al., 1987] and neutrophils [Harris et al., 1984].

Collagenases produced by fibroblasts and alveolar macrophages are about equally active against collagens I and III, while collagenases synthesized by neutrophils degrade collagen I much more rapidly than collagen III [Horwitz et al., 1977]. Collagenase can be activated by oxidative processes [Weiss et al., 1989] and has been detected in bronchoalveolar lavage fluid of patients in idiopathic pulmonary fibrosis and in sarcoidosis [Gadek et al., 1979; O'Connor et al., 1988]. However others have found that fibroblasts obtained from fibrotic subjects contain less collagenase activity compared to controls [Selman et al., 1991]. Collagenase synthesis is increased by IL-1, TNF and PDGF [Raghu and Kinsella, 1993]. Other collagen degrading proteases of the lung include neutrophil elastases, cathepsin G and gelatinase [Gadek et al., 1980; Janoff, 1985; Vogelmeier et al., 1991] and macrophage elastases [Senior et al., 1989]. Proteases are believed to play an important role in emphysema [Janoff, 1985] and bronchitis [Stockley et al., 1983].

3.4.3 Type III procollagen peptide

Collagen type III is a homotrimer composed of three $\alpha 1$ chains. The N-terminal propeptide of the procollagen can be detected in bronchoalveolar lavage fluid and in serum. In areas of early fibrosis more type III collagen than type I collagen is found while in later stages of fibrosis, type I predominates [Bateman et al., 1981; Raghu et al., 1985]. In a bleomycin model of fibrosis, type III procollagen mRNA is increased in individual cells of affected areas, measured in situ [Shahzeidi et al., 1993]; In later study also increased collagen I was observed, preceding the increases in type III procollagen mRNA [Shahzeidi et al., 1993]. Altered ratios of collagen I and collagen III that have been observed during development of fibrosis may be explained by three different mechanisms, i.e. (1) a switch of fibroblasts that initially synthesize collagen III and later switch to collagen I production, (2) proliferation of subsets of fibroblasts with distinct production of type I and III collagens, or (3) increased degradation rate of collagen III compared to type I collagen [Raghu and Kinsella, 1993]. N-terminal type III procollagen peptides (PIIIP) have been measured in bronchoalveolar lavage or serum of subjects with fibrotic lung disease including idiopathic pulmonary fibrosis [Low et al., 1983; Cantin et al., 1988] and asbestosis [Okazaki et al., 1983; Cavalleri et al., 1991]. Previously, serum PIIIP was found to be related to radiological stage of CWP [Janssen et al., 1992a]. Correlations between serum and lavage PIIIP levels have been studied by Low and coworkers [Low et al., 1989; Low et al., 1992] and serum PIIIP has been forwarded as a marker of early clinical diagnosis in fibrotic lung disease [Low et al., 1992; Janssen et al., 1992] and/or mineral dust exposure [Cavalleri et al., 1993; Becklake et al., 1993].

Chapter 4

DESIGN OF THE STUDIES

4.1 Description of the study population and of the biomarkers evaluated

In line with biological mechanisms involved in mineral dust induced lung disorders (chapter 3), the specific studies in this thesis are subdivided into three parts. In separate chapters (1) oxidant/antioxidant parameters, (2) cytokines, and (3) type III collagen will be discussed in view of their applicability as biological markers. As already outlined for some of the parameters their candidacy as biomarkers of CWP was forwarded from previous cross-sectional surveys by our laboratory. Three study groups of miners from the Belgian coal mining region "Kempen" were recruited for a follow-up. In the first study, spontaneous and stimulated release of TNF by peripheral blood monocytes was measured in 66 coal workers and 12 controls. Monocytes were stimulated with coal dust, silica and endotoxin (LPS), and the release was related to severity of CWP based on chest radiograph classification [Borm et al., 1988]. In the second study, antioxidants in red blood cells (GSH, GSSG, GST, Gpx, SOD) and serum (Vitamins A and E) of 73 coal workers and 16 controls were analysed and related to chest radiographs [Engelen et al., 1990; Evelo et al., 1993]. The third study described measurement of serum PIIP in a subgroup of the latter study group [Janssen et al., 1992]. The evaluation of these parameters in a the follow-up design are discussed in chapter 7 (antioxidants), chapter 8 (TNF), and in the chapters 11 and 12 (PIIP). New markers in the follow-up concerning cytokine-related mechanisms include plasma soluble TNF receptors (sTNF-R55 and sTNF-R75) (chapter 9) and monocyte release of TGF β and IL-6 (chapter 10). With regard to oxidative stress responses, serum total radical antioxidant parameter (TRAP) (chapter 5) as well as oxidative DNA damage (8-oxodG) in lymphocytes (chapter 6) are evaluated. Figure 4.1 shows how the markers evaluated in this thesis should be seen in the pathogenesis of mineral dust induced lung disorders as outlined in chapters 2 and 3.

The particular choice of new parameters was made in view of their relation with the markers previously determined (TNF, AOE), in order to elucidate their possible involvement in coal dust induced lung disorders (chapter 3) as well as their candidacy as biomarkers in mineral dust exposure and disease. Both soluble TNF receptors (R55 and R75) are determined in the plasma of coal workers because of their natural occurrence in human plasma and the proposed regulatory role in the systemic effects of TNF [Jäätelä, 1990; Ulich et al., 1993], and their antifibrogenic effects observed in mineral dust induced fibrosis [Piguet et al., 1994]. Monocyte release of TGF and IL-6 are included, since in line with TNF (1) they are both released from coal dust stimulated alveolar macrophages [Gosset

Figure 4.1 The integration of biological mechanisms that have been used in this thesis for the generation and testing of biomarkers for mineral dust induced health effects. Markers are indicated in rectangulars. The two triangles indicate the main balances that can be tipped during ongoing inhalation and deposition of dust particles. Disturbance of the balance between reactive oxygen species (ROS) and antioxidants may result in oxidative stress responses [Janssen et al., 1993]. Proinflammatory cytokines may trigger cells and tissue to become activated, proliferated and/or differentiated, while anti-inflammatory cytokines may act as feedback mechanisms to these effects [Kelley, 1990]. Both pathways are interrelated, since ROS generating cells (e.g. neutrophils) may be recruited by cytokines, cytokines are known to modulate antioxidant expression and activities within cells, and ROS may either amplify or counteract cytokine responses. Both oxidative and cytokine mediated pathways play a role in a number of lung disorders including alveolitis, fibrogenesis, lung function decline, chronic bronchitis, and cancer. During the development or progression of these disorders, both balances may be affected, and thus likely modify pathological responses. In this cascade of events, presented markers may reflect (chronic) exposure to mineral dust, (non)pneumoconiotic pathogenesis resulting from this exposure, and/or - both in the absence or presence of disease- individual differences in susceptibility to mineral dust induced lung disorders.

et al., 1991; Vanhe et al., 1994], and (2) their presence is demonstrated in lungs of fibrotic patients [Broekelman et al., 1991; Vanhe et al., 1995]. Formation of 8-oxodG in lymphocyte DNA is determined in order to study whether the changes in antioxidant status observed in animal models of mineral dust exposure [Janssen et al., 1994] as well as in humans [Borm et al., 1988] may cause significant oxidative effects in subjects chronically exposed to mineral dusts. Furthermore, measurement of oxidative DNA damage in the present study group (i.e. in exposed subjects plus pneumoconiotic subjects) could provide insight in the role of the proposed carcinogenicity in existing pneumoconiotic disease [McDonald, 1989]. TRAP was determined to evaluate the role of plasma as antioxidant pool to cope with pathogenic effects caused by reactive oxygen species in the lung, and to study their relation with antioxidants previously measured in coal workers [Borm et al., 1988].

Although specific hypotheses have been generated for specific candidate markers, the follow-up of these cohorts was carried out with a number of general purposes:

- (1) Determination of development and progression of pneumoconiosis during the follow-up period by longitudinal evaluation of chest radiographs.
- (2) Renewed measurement of TNF, PIIP and antioxidants (AOE) to determine five-year changes in these markers in relation to changes in disease stage and reproducibility of the cross-sectional observations.
- (3) Inclusion of new candidate markers (TGF, IL-6, TRAP, 8-oxodG) and cross-sectional and/or retrospective data analysis of these parameters.
- (4) Inclusion of accurate exposure assessment (i.e. cumulative dust exposure) to investigate the role of exposure in relation to all parameters, including candidate markers and disease (progression).
- (5) Inclusion of lung function data and respiratory complaints to assess for nonpneumoconiotic disease and/or confounders.
- (6) Inclusion of HRCT in a subgroup, to detect and quantify early stage fibrosis.
- (7) Comparison of markers to a carefully selected control group with known occupational and medical history and lifetime smoking habits.

4.2 Peripheral blood versus the lung

Bronchoalveolar lavage or lung biopsies would be ideal methods to screen for biomarkers in relation to the "target tissue" studied. However, validated biomarkers should be easily applicable in large scale routine surveys, i.e. the biomarkers should be minimally invasive or noninvasive [Weill, 1993].

Although, most interactions and the eventual effects comprise the lung tissue, all markers evaluated in this thesis are measured in the peripheral blood obtained by venapuncture. The use of peripheral blood is underscored by the following observations:

- (1) Blood sampling is a relative non-invasive method to retrieve cells and fluid, in comparison to bronchoalveolar lavage.
- (2) Red blood cells are considered as important antioxidant carriers for the lung [van Asbeck et al., 1984; Engelen et al., 1990], and since mature erythrocytes contain no nucleus or organelles they are not capable of restoring their antioxidant levels by *de novo* synthesis of lost proteins.
- (3) Reduced antioxidant levels (vitamin A, C, E) in human plasma are considered as risk factors in lung disease [Morabia et al., 1989; Schwartz and Weiss, 1994; Britton et al., 1995].
- (4) Peripheral blood lymphocytes may be considered as a surrogate tissue for target cells from which DNA may be obtained relatively non-invasive.
- (5) Peripheral blood monocytes are recruited to the lung at inflammatory sites and differentiate into mature alveolar macrophages. Increased recruitment of monocytes from the blood (by chemotactic factors) is known to occur in pneumoconiotic disease (see chapter 3).
- (6) In contrast to alveolar macrophages, peripheral blood monocytes are not directly exposed to particles and toxicants in the lung. Ex-vivo stimulation of monocytes is thus less likely to be biased by *in vivo* stimulatory artefacts.
- (7) Cytokine release as well as mechanisms leading to increased cytokine release in the lung may be involved in extrapulmonary effects by means of priming mechanisms [Mohr et al., 1991; Mohr et al., 1992]. Soluble cytokine receptors released from blood cells may comprise a major systemic source to either reduce or amplify these effects.
- (8) Procollagen peptides are abundant in human plasma and are specific due to collagen metabolism, and correlations between PIIP in lavage fluid and serum have been reported in fibrotic lung disease [Low et al., 1983].

PART 1
Oxidative damage and
antioxidant defense

Chapter 5

SERUM TOTAL RADICAL-TRAPPING ANTIOXIDANT PARAMETER (TRAP) IN COAL WORKERS.

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Abstract

Formation of reactive oxygen species in response to (mineral) dust exposure is a well-described phenomenon in processes ultimately leading to lung fibrosis. We determined serum total radical-trapping antioxidant parameter (TRAP) and total red blood cell glutathione in coal miners with or without coal workers' pneumoconiosis (CWP) and in non-dust-exposed control subjects. Total serum antioxidant capacity was significantly increased in new cases of coal workers' pneumoconiosis and was inversely related to individual cumulative exposure in cases without "active" fibrosis. These findings are in line with the hypothesis that oxidative stress is present in the lung during active phases of coal workers' pneumoconiosis.

Introduction

Reactive oxygen species (ROS) play an essential mediating role in the damage to many cell types in the lung in response to inhalation of mineral dusts (asbestos, silica, coal). Both physical and chemical surface properties of the inhaled dust particles and oxidative burst by polymorphonuclear granulocytes, alveolar macrophages and other inflammatory cells are associated with the formation of free radicals such as $\text{OH}\cdot$, H_2O_2 and $\text{O}_2^{\cdot-}$ [Borm et al., 1990; Janssen et al., 1992b]. In concert with other biological mechanisms such as fibroblast activation, chemotaxis and secretion of cytokines, ROS and lysosomal enzymes may ultimately lead to pulmonary fibrosis. Fortunately, the lung possesses an elaborate defence systems against ROS, commonly summarized under the name antioxidant system (AOS) [Janssen et al., 1992b].

In the course of our studies seeking to explain why certain coal workers develop coal workers' pneumoconiosis (CWP) whereas others -equally exposed-

do not [Borm et al., 1990] we observed that many components of the AOS were related to the stage of pneumoconiosis [Engelen et al., 1990]. Previously, most of the AOS markers were measured in red blood cells, with only vitamins A and E being measured in serum. The aims of the present study were to determine the total radical-trapping antioxidant parameter (TRAP) in serum of coal workers and to test consistency with our previous findings.

Subjects, materials and methods

Participants in this study were 104 coal miners from our previous cohorts. Informed consent was obtained, and the study was approved by the ethical standards of our institution's responsible committee according to the Helsinki Declaration of 1975, as revised in 1983. Pneumoconiotic stages of both the original cross-sectional studies [Borm et al., 1990; Engelen et al., 1990] and the follow-up, were determined by chest x-ray classification according to the International Labour Organization (ILO) criteria by a panel of three physicians. Five-year development (among 80 reference miners) or progression (in 24 miners with CWP) was deduced from paired readings of each individual's 1987 and 1992 chest x-rays. In addition 26 age matched male controls without any occupational dust exposure were randomly selected from the general population in the same area. Subjects with a known history of pulmonary diseases or current allergies or chronic diseases of liver, kidney, or bladder were excluded. Smoking habits and medical history were obtained from questionnaires. Occupational exposure was defined as years underground and as cumulative dust exposure. Cumulative exposure, expressed as hours exposed $\times$ grams of dust per cubic meter sampled air, was estimated from each individual's job history and from dust measurements at various sites of the coal mine.

Total glutathione in red blood cells was measured according to Anderson [Anderson, 1985]. Serum TRAP was measured as originally described by Wayner et al. [Wayner et al., 1987]. Briefly, to a closed compartment at 37°C containing an oxygen electrode (YSI Model 5331 Probe; YSI Model 3500 Biological Oxygen Monitor; Yellow Springs Instruments, Yellow Springs, OH), we added 15 μ L of serum mixed with linoleic acid (25/1, by vol; Aldrich Chemical Co., 99%, cat. no. 22.392-7) and 15 of 2,2'-Azobis(2-amidinopropane) HCl (0.25 mol/L; Polysciences, cat. no. 08963) to 1.5 mL Phosphate Buffered Saline (140 mmol/L NaCl, pH=7.4). After the interval of inhibited peroxidation (i.e., serum lagtime), we added 25 μ L of a water-soluble vitamin E-analog, Trolox (6-hydroxy-2,5,7,8 tetramethyl-chroman-2-carboxylic acid), (133mmol/L; Aldrich Chem. Co., 97%, Cat. no. 23.881-3) as a standard (i.e., Trolox-lagtime). TRAP is defined as the amount of peroxy radicals captured non-enzymatically per liter of solution:

$$\text{TRAP} = (\text{T-serum} / \text{T-trolox}) \cdot n \cdot f \cdot [\text{Trolox}]$$

where, T-serum is the lag time of the serum sample; T-trolox is the lag time of Trolox; *n* is a stoichiometric factor, indicating the number of radicals (mol) captured by an antioxidant (i.e., 2.0 for Trolox); and *f* is the dilution factor of serum vs Trolox.

Table 5.1 Characteristics of the control and study groups, and corresponding measures of red blood cell glutathione and serum TRAP.

	Controls	Reference miners		Miners with CWP	
		NP	P	NP	P
<i>n</i>	26	76	4	11	13
Age, years	50.5(1.01) ^a	47.7(0.57)	44.5(2.84)	50.0(1.98)	52.1(1.13) ^a
Exposure					
Underground, years	-	22.3(0.44)	23.8(2.84)	23.7(0.95)	25.8(1.41)
Cumulative dust, gh/m ³	-	89.6(6.4)	130.0(23.7)	127.4(13.3)	132.6(16.7)
Glutathione, mmol/gHb	6.03(0.18)	5.72(0.11)	6.15(0.46)	5.81(0.34)	6.09(0.28)
TRAP, μmol/L	924(25) ^b	926(31) ^b	1108(66) ^c	944(52)	876(28)

Values are mean (SEM): P = progression (or development) of pneumoconiosis; NP = no progression.

^a Significantly different from reference miners (*P* < 0.05, Kruskal-Wallis).

^b TRAP measured in only 5 controls and 13 NP-reference miners.

^c Significantly different from all other groups (*P* < 0.05, Kruskal-Wallis).

Results

No significant differences in TRAP concentrations (Table 5.1) were observed between non-dust-exposed controls (*n* = 5), reference miners (*n* = 13) and miners with CWP (*n* = 28) (*P* > 0.7, ANOVA). The CWP group showed an inverse relation between serum TRAP and pneumoconiotic stage (Spearman's ρ = -0.39; *P* < 0.05); however, the variation in TRAP was large, so that differences between the stages were not significant (*P* = 0.22). Although the same trend is seen in TRAP and glutathione data, there was no significant correlation between serum TRAP and red blood cell glutathione concentrations (*n* = 46, *r* = 0.016, *P* > 0.9).

During this 5-year follow-up, either several subjects developed CWP newly or there was progression of existing CWP. Subjects with newly developed pneumoconiosis (*n* = 4) had significantly higher TRAP concentrations than the control miners who did not develop CWP (Table 5.1). Glutathione levels were not significantly increased in those subjects but showed a similar trend.

Interestingly, TRAP concentrations and cumulative dust exposure were correlated in the miners group with CWP, but this was significant only in the non-progressed individuals ($n = 11$, $r = -0.6$, $P < 0.05$).

Discussion

Previously we demonstrated a relationship between several factors of the antioxidant defense system and pneumoconiotic stage [Engelen et al., 1990; Evelo et al., 1993]. We observed that red blood cell glutathione concentration [Engelen et al., 1990] as well as red blood cell glutathione-S-transferase activity [Evelo et al., 1993] were decreased in coal miners at early stages of CWP (ILO classification 0/1-1/2). On the other hand, glutathione peroxidase activity in red blood cells was increased in the early stages of CWP [Engelen et al., 1990]. From these findings, we hypothesized that the variations in red blood cell antioxidant factors reflected the oxidative stress imposed by the inflammatory processes in the lung [Borm et al., 1990]. The present data failed to confirm the decrease in (total) glutathione in red blood cells in the early stage of CWP; the fact that miners in this study were all retired and therefore had not been exposed for at least the preceding 2 years might explain this discrepancy. In new cases of CWP and those miners whose disease progressed over 5 years higher concentrations of total glutathione were observed. Interestingly, total serum antioxidant capacity undergoes changes similar to red blood cell glutathione, although there is no correlation between these two markers on an individual basis. Moreover, TRAP appeared to be inversely related to individual (cumulative) exposure but only in miners without "active" fibrosis. Because TRAP is the end result of several antioxidants, we have no information on the causal factor(s) involved but consider vitamin A and E unlikely candidates because of our previous results [Engelen et al., 1990]. The present findings are consistent with our previous hypothesis that during the first phases or progression of CWP, oxidative stress is present in the lung. Either a decrease or increase in anti-oxidant factor(s) can be observed, the change being dependent on the relative impacts of (a) the extra load on the antioxidant system and (b) the extra synthesis of antioxidant factors.

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Chapter 6

OXIDATIVE DNA DAMAGE IN PERIPHERAL BLOOD LYMPHOCYTES OF COAL WORKERS.

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Abstract

Reactive oxygen species are important mediators of both mineral dust induced (malignant) lung disease and *in vitro* DNA-damage. Therefore, we studied *in vivo* oxidative DNA-damage in coal workers who had been chronically exposed to silica-containing dust. In peripheral blood lymphocytes of 38 retired coal miners (eight with coal workers' pneumoconiosis, 30 references) and 24, age matched, non-dust-exposed controls 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) was determined by reversed phase high-performance liquid chromatography (HPLC) with electrochemical detection (ECD). The ratio of 8-oxodG residues to deoxyguanosine (dG) was related to individual cumulative dust exposure estimates and pneumoconiotic stage as established by chest radiography. The ratio of 8-oxodG to dG ($\times 10^{-5}$) in lymphocytes did not differ between miners with coal workers pneumoconiosis (2.61 ± 0.44) or miners without coal workers' pneumoconiosis (2.96 ± 1.86). However, oxidative DNA-damage in all miners was higher than in the non-dust-exposed controls (1.67 ± 1.31). 8-oxodG/dG was not related to individual cumulative coal dust exposure, age or smoking (pack-years) when evaluated by multiple linear regression. We suggest that oxidative damage to the DNA of peripheral blood lymphocytes may be introduced by increased oxidative stress responses in subjects chronically exposed to mineral dusts. Whether this is an important pathway in the suggested carcinogenicity of silica is still an open question.

Introduction

It is well known that occupational exposure to mineral dusts can lead to the development of fibrotic lung diseases (i.e. asbestosis, silicosis, coal workers pneumoconiosis). Many studies have demonstrated that reactive oxygen species (ROS) are involved in the biological effects of these mineral dusts [Mossman and Marsh 1991; Janssen et al. 1993]. Both physical and chemical surface properties of the inhaled dust particles and the oxidative burst by polymorphonuclear

granulocytes, alveolar macrophages and other inflammatory cells are associated with the formation of free radicals such as $\text{OH}\cdot$, H_2O_2 and $\text{O}_2^{\cdot-}$ [Janssen et al. 1993]. The involvement of elaborate antioxidant defence systems against the harmful effect of ROS, has been studied by our laboratory to explain for differences in susceptibility to the development and progression of Coal Workers Pneumoconiosis (CWP) in coal workers [Borm et al. 1986; Engelen et al. 1988; Evelo et al. 1993; Schins et al. 1994a] and in experimental fibrosis in rats after *in vivo* exposure to asbestos and cristobalite [Janssen et al. 1992b, 1994a].

On the other hand, chronic inhalation of mineral dusts, especially asbestos and to a lesser degree silica, has also been associated with the development of lung cancer [McDonald 1989; Mossman et al. 1990; Holland 1990; Craighead 1992]. Since ROS are important mediators of *in vitro* DNA-damage, and related to carcinogenesis [Trush and Kensler 1991; Janssen et al. 1994b], it is suggested that oxidant generation may also play a crucial role in carcinogenic action of mineral dusts [Ghio et al. 1990; Janssen et al. 1993]. A particular type of DNA-damage which is directly related to the action of ROS, is the C8 oxidation of deoxyguanosine (dG). The resulting 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) may be used as a biological marker of oxidative stress to DNA [Kasai et al. 1986; Kuchino et al. 1987]. Recently, Hanaoka *et al.* [1993] have measured 8-oxodG/dG ratios in lymphocytes of asbestosis patients, to study the *in vivo* significance of the oxidative stress believed to be involved during asbestos exposure and in asbestosis. In the present study we describe the determination of 8-oxodG/dG ratios in peripheral blood lymphocytes of coal miners with or without CWP and in controls not occupationally exposed to dust. The purpose of the study was to evaluate 8-oxodG/dG analysis in peripheral blood lymphocytes as a biological marker of the cumulative (silica-)dust exposure or on the other hand of the fibrotic effects (pneumoconiosis) which result from chronic coal dust exposure.

Materials and methods

As a part of a prospective cohort study among coal workers [Borm 1994], the ratio of 8-oxodG/dG was determined in peripheral blood lymphocyte DNA of coal miners ($n=38$) and non-dust-exposed controls ($n=24$). Prior to the blood sampling, a questionnaire, including informed consent was obtained from each participant. On the day of blood sampling, a chest radiograph was made of each miner, and a personal interview to confirm answers in the questionnaires and to check the medical files and job history. All subjects were classified as smokers, nonsmokers, former smokers or never smokers. Amount smoked was expressed in pack-years as described previously [Jorna et al. 1994a], after verification of smoking reporting during the personal interview and comparison with data from the previous cross-sectional studies [Borm et al. 1990] to minimize

reporting bias. The same was done for medical history. Pneumoconiotic staging of the coal miners was achieved by classification of chest radiographs by three occupational physicians according to a standard protocol of the International Labour Organization [I.L.O. 1980] and has been described elsewhere [Schins et al. 1994b]. The cumulative dust exposure was determined from job-exposure matrices as described previously [Jorna et al. 1994a].

Isolation of DNA from peripheral blood lymphocytes

Peripheral blood lymphocytes were isolated as follows: blood (3 x 10 ml, EDTA tubes) was sampled, stored at 4°C, and subsequently left overnight (20.00 h p.m.-8.00 h a.m.) at room temperature. Lymphocytes were then isolated by buoyant density centrifugation (Lymphoprep-Nycomed), adjusted to concentrations of 5×10^6 cells/ml and seeded in portions of 0.5 ml to tissue culture dishes (Costar TC-24) to allow adherence during 30 minutes incubation in RPMI 1640 supplemented with 10% FCS. All nonadjacent cells (repetitive agitation and washing) from each subject were pooled, washed twice in PBS (10 min., 800 g) and stored at -70°C until isolation of cellular DNA according to the method of Gupta [Gupta 1984], as described previously [Schilderman et al. 1993]. Briefly, cells were homogenized in 1% SDS/1 mM EDTA and subsequently incubated with proteinase K. The homogenate was successively extracted with 1 vol of phenol, 1 vol of phenol/chloroform/isoamyl alcohol (25/24/1; v/v/v) and 1 vol of chloroform/isoamyl alcohol (24/1). After addition of 0.1 vol of 3M NaAc (pH=6.0), nucleic acid was precipitated with 2 vol of ice-cold ethanol, washed with 70% ethanol to remove salt and dissolved in 5 mM Tris-HCl (pH=7.4) and 1mM EDAT (pH=7.4). RNA was destroyed by addition of RNase T1 (150U/mg DNA) and RNase A (300 µg/mg DNA) during 30 min of incubation at 38°C. After extraction of the digest with 1 vol chloroform/isoamyl alcohol (24/1), DNA was recovered as described above and dissolved in 5mM Tris-HCl (pH=7.4). DNA concentration was assayed spectrophotometrically. The DNA was digested to deoxynucleosides by treatment with nuclease P1 (25 U/mg DNA) and alkaline phosphatase [Lutgerink et al. 1992]. DNA was isolated from lymphocytes of controls and miners in randomised order. Samples with a DNA yield below 40 µg were excluded from analysis. DNA yield did not differ between miners and controls and not related to day of isolation.

HPLC/ECD analysis of 8-oxodG

The ratio of 8-oxodG/dG was determined according to Floyd *et al.* [1986] as described previously [Schilderman et al. 1993]. Briefly, 8-oxodG was detected by HPLC/ECD analysis, using a Spectroflow 480 Solvent delivery system coupled with a Kratos spectroflow 783 programmable absorbance detector and an Antec electrochemical detector CU-04-AZ (850 mV). A supelcosil LC-18S column (supelco: 250 x 4.6 mm) was used in conjunction with a 5µm diameter guard

column. The mobile phase consisted of 10% aqueous methanol containing sodium acetate (25 mM), citric acid (12.5 mM), NaOH (30 mM) and acetic acid (10 mM). Elution was performed at a flow rate of 1.0 ml/min. The lower limit of detection was 40 fmol absolute for 8-oxodG, or 1.5 residue/10⁶ dG requiring a minimum yield of 35 µg DNA per sample. dG was simultaneously monitored at 260 nm. Oxidative DNA damage was expressed as the ratio of 8-oxodG to dG. Samples were analyzed randomly with a calibration curve and a selection of controls on each run. 8-oxodG/dG ratios were not dependent on day of isolation, day of HPLC analysis, or DNA yield.

Statistical evaluations

Study group characteristics were evaluated by the Student's t-test and the non parametrical Mann-Whitney U-test, i.e. age, pack-years smoked, exposure and oxidative DNA damage. The χ^2 -test was used to evaluate smoking and medication. Relations between 8-oxodG/dG and age, pack-years, and exposure parameters (i.e. miners only) were evaluated by (multiple) linear regression models (stepwise regression analysis). Statistical evaluation was made using STATGRAPHICS version 6 (Manugistics Inc., Rockville Maryland 20852. USA).

Results

Fig. 6.1 illustrates that 8-oxodG/dG ratios in the miners were significantly higher than in the non-exposed controls ($p<0.01$). All other group characteristics and ratio's of 8-oxodG/dG are shown in Table 6.1.

Figure 6.1 Ratio of 8oxodG/dG in peripheral blood lymphocytes of 24 non-dust-exposed controls and of 38 coal miners

Although miners with CWP were significantly more exposed on average (see Table 6.1), 8-oxodG/dG ratio's were not significantly different for miners without CWP versus miners with CWP. On the other hand, oxidative DNA-damage was significantly lower in the non-dust-exposed control group versus reference miners. Although age in the control group was significantly higher, correlation between age and oxidative DNA-damage was observed neither in any of the subgroups nor in the total study group. The calculated cumulative exposure (to respirable coal dust as well as quartz content) was significantly higher in the miners with CWP compared to reference miners, but years exposed, time elapsed since first exposure and time retired were not (Table 6.1). However, none of the exposure parameters was related to oxidative DNA damage in a stepwise multiple linear regression. No relation was observed between stage of pneumoconiosis and oxidative DNA damage (data not shown), nor was oxidative DNA-damage affected by medication.

Table 6.1 Study group characteristics and ratios of 8oxodG to dG in peripheral blood lymphocytes of miners with coal workers pneumoconiosis, reference miners and a non-dust-exposed control group.

	non-exposed controls	reference miners	miners with CWP
	n = 24	n = 30	n = 8
age (years)	50.7 (1.1)	46.6 (0.9) #	46.5 (2.1)
smoking (pack-years)	105.6 (18.6)	92.9 (16.4)	140.4 (37.9)
years underground	-	22.1 (0.8)	24.6 (1.7)
years since first exposure	-	28.1 (1.1)	29.1 (1.9)
months retired	-	44.4 (3.4)	51.5 (2.7)
cumulative exposure			
dust (ghm ⁻³)	-	92.6 (11.7)	144.9 (11.8) *
quartz (ghm ⁻³)	-	4.90 (0.56)	7.42 (0.61) *
8-oxodG/dG (x10 ⁻⁵)	1.67 (0.27)	2.96 (0.34)#	2.61 (0.44)

All values (i.e. mean and standard error) are evaluated by the Mann-Whitney U test: *p<0.05 significantly different from reference miners; #p<0.01 significantly different from non-dust-exposed controls; CWP = miners with coal workers' pneumoconiosis (ILO chest radiograph >0/0).

No differences in amount of smoking were present among the three groups, and no correlation was observed between 8-oxodG/dG ratios and pack-years in any of the subgroups or in the total group. The effects smoking on the 8-oxodG/dG ratios are also shown in Table 6.2. Oxidative DNA-damage did not differ between current smokers and nonsmokers, or between former smokers and never smokers. No synergistic or antagonistic effects of smoking plus dust exposure on

8-oxodG/dG ratios were observed (Table 6.2). Multiple regression (stepwise analysis) did not result in any significant relation between any parameter of smoking, exposure, age or medication and the ratio of 8-oxodG/dG.

Table 6.2 Effects of smoking on the ratio of 8-oxodG/dG ($\times 10^{-5}$) in peripheral blood lymphocytes of controls and coal miners.

	non-exposed controls (n=24)	reference miners (n=30)	miners with CWP (n=8)	all subjects (n=62)
smokers	1.31 (0.28) (n=11)	2.86 (0.65) (n=10)	3.14 (0.85) (n=4)	2.26 (0.36) (n=25)
non smokers	1.96 (0.44) (n=13)	3.01 (0.40) (n=20)	2.07 (0.11) (n=4)	2.54 (0.28) (n=37)
ever smokers	1.55 (0.20) (n=20)	3.02 (0.40) (n=24)	2.71 (0.50) (n=7)	2.40 (0.23) (n=51)
never smokers	2.23 (1.37) (n=4)	2.72 (0.57) (n=6)	1.87 (-) (n=1)	2.47 (0.55) (n=11)

All values (i.e. mean and standard error) are evaluated by the Mann-Whitney U test at $p=0.05$; CWP = Coal workers' pneumoconiosis (ILO chest radiograph $>0/0$).

Discussion

In the present study we evaluated the ratio of 8-oxodG to dG residues in peripheral blood lymphocytes of coal workers, and controls not occupationally exposed to (mineral) dusts. Oxidative DNA damage was found to be significantly higher in lymphocytes of coal miners without pneumoconiosis than on those of the non-exposed controls. A similar study by Hanaoka *et al.* [1993] studying 8-oxodG/dG ratios in lymphocytes of asbestosis patients did not identify differences with subjects without asbestosis. However, their control group was selected from patients from the same hospital. Moreover, all asbestosis patients studied by Hanaoka *et al.* [1993] had radiological evidence of fibrosis (chest radiograph findings of 1/0 - 2/1 according to ILO criteria [ILO, 1980]). In contrast, the majority of the coal workers in our present study (30/38) were not fibrosis patients. In our study lymphocyte 8-oxodG/dG ratios were not related to smoking habits, age or medication, nor was there a synergistic effect between smoking and occupational exposure. This indicates that the chronic inhalation of mineral dust is the major factor responsible for the observed difference in oxidative DNA damage between miners and controls. However, individual 8-oxodG/dG ratios of coal miners did not correlate with cumulative exposure estimates or to the presence of fibrotic

lesions visible by chest radiography. Therefore, we suggest that either the presence or absence of a history of long term heavily occupational exposure to mineral dusts may account for a all-or-none significant contribution to lymphocyte DNA damage.

Several authors have shown that mineral dusts are able to increase oxidative damage in (isolated) DNA [Leanderson et al. 1988; Adachi et al, 1994; Takeuchi and Morimoto 1994]. Recently, Daniel et al. [1994] showed that silica can induce strand breaks in isolated DNA. Effects of silica have been described on sister chromatid exchanges [Pairon et al. 1990], in cell transformation assays and micronucleus assay [Hesterberg et al. 1986]. Interestingly, Pairon *et al.* showed that sister chromatid exchange frequencies in lymphocytes were invreased upon exposure to tridymite only if monocytes were also present in the culture dishes [Pairon et al. 1990]. Although less clear results were observed with silica, the authors suggested that a phagocytotic action of phagocytotic cells (monocytes) and the subsequent signaling mechanisms between these cells and the target cells (lymphocytes) are necessary to induce cytogenetic effects. Similarly, signaling between pulmonary cells (macrophages, epithelial cells, fibroblasts) in the presence of coal dust and peripheral blood lymphocytes may be the reason for the increased 8-oxodG levels observed in the lymphocytes of the miners. Recently, Driscoll et al. [1994] have presented *in vivo* support for a mutagenic action of silica. They showed that upon silica inhalation, rat alveolar epithelial cells have increased mutagenicity (HPRT locus mutations), and even more interestingly, that cultured rat epithelial cells also show an increased mutagenic response upon incubation with bronchoalveolar lavage cells from these silica-exposed rats [Driscoll et al. 1994]. However, in contrast to studies on direct interactions between mineral dust and DNA [Leanderson et al. 1988; Adachi et al, 1994; Janssen et al. 1994b], it is not clear whether or not ROS and antioxidant status play a significant role in the indirect genotoxic action of silica observed by Pairon et al. [1990] and Driscoll et al. [1994].

Previously, we have reported that antioxidant factors in the blood may be changed by chronic silica exposure or by the presence of silicotic lesions [Borm et al. 1986; Engelen et al. 1988; Evelo et al. 1993; Schins et al. 1994a]. Unfortunately, we have no data on antioxidant protection of lymphocytes in these workers, but neither red blood cell glutathione, glutathione-S-transferase and glutathione reductase, nor plasma total radical trapping capacity (n=13) [Schins et al. 1994a] were related to oxidative DNA damage (data not shown). However, our data do show that peripheral blood cells in (healthy) coal miners with a long working history [Borm et al. 1986; Borm et al. 1990; Schins et al. 1994c] behave and respond differently in comparison to cells of subjects without a mining history. We suggest that oxidative damage to the DNA of peripheral blood lymphocytes may be introduced by increased oxidative stress responses in the lung in subjects

chronically exposed to mineral dusts. Whether this is an important pathway in the suggested carcinogenicity of silica is still an open question.

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Chapter 7

BLOOD ANTIOXIDANT STATUS IN COAL DUST INDUCED RESPIRATORY DISORDERS: A LONGITUDINAL EVALUATION OF MULTIPLE BIOMARKERS.

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Abstract

To investigate the involvement of oxidative stress in coal dust induced respiratory disorders, red blood cell and serum antioxidants in 66 coal miners were related to five-year changes in coal workers pneumoconiosis (CWP), chronic bronchitis, and lung function decrease ($n=40$). Reduced (GSH) and oxidized (GSSG) glutathione concentrations, glutathione peroxidase (Gpx), glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase activities were measured in erythrocytes and Vitamin A, Vitamin E and iron were determined in serum. Changes in CWP were determined by chest radiography, chronic bronchitis was determined from a validated questionnaire and lung function decline was calculated by linear regression for a ten year interval before blood sampling. SOD activity was increased in miners with progression of CWP (2308 ± 156 vs 1703 ± 155 U/gHb, $p < 0.05$), and GSH was reduced in those with chronic bronchitis at follow-up (3.53 ± 0.16 vs 4.05 ± 0.09 mmole/gHb, $p < 0.01$). Stepwise discriminant analysis showed that for both pneumoconiotic and nonpneumoconiotic respiratory disease in this cohort, increased enzymatic antioxidants (i.e. Gpx, Catalase, SOD) were high risk factors, while increased "nonenzymatic" antioxidants (i.e. Vitamin E, GSH) indicated reduced risk. GST activity showed discriminative power in two ways, i.e. decreased activity in those at risk for CWP, but increased in those at risk to obstructive disease. We conclude that the multiple marker approach applied here, shows the relevance of interpretation of total "antioxidant status" versus single antioxidant measurements in health screening of individuals at risk for respiratory impairments.

Introduction

It is well known that occupational exposure to coal dust can lead to coal workers' pneumoconiosis [Crystal et al. 1991]. However, chronic inhalation of coal dust

may also cause other respiratory effects such as emphysema, chronic bronchitis and airflow obstruction [Wouters et al. 1994]. The role of oxygen radicals in these diseases has been discussed extensively over the last decade [Cantin and Crystal 1985, Barnes 1990, Kehrer 1993, Halliwell and Cross 1994]. Because of the intrinsic physicochemical characteristics of mineral dusts and their complex behaviour in the lung, the significance of "oxidative stress" in the development of mineral dust related respiratory disorders has received special attention [Kamp et al. 1992, Janssen et al. 1992]. Previously, we showed that many components of the blood antioxidant system of coal miners were related to the severity of coal workers pneumoconiosis (CWP) [Engelen et al. 1990]. More recently, altered antioxidant status in coal dust exposure and stage of pneumoconiosis was also reported by others, in the blood [Perrin-Nadif et al. 1996], and in bronchoalveolar lavage [Vallyathan et al. 1995]. Since antioxidant status has also been related to obstructive disease [Morabia et al. 1989, Schwartz and Weiss 1994, Britton et al. 1995], one could suggest that the impaired oxidant/antioxidant balance observed in coal workers [Engelen et al. 1990] may also play a role in the nonpneumoconiotic respiratory effects in these subjects.

Although the presence of "oxidative stress" in coal dust exposed subjects may be evident, its significance in the development or progression of coal dust induced respiratory disorders remains to be elucidated. To determine the significance of antioxidant measurements with regard to the development or progression of a disease, a longitudinal design is necessary [Schulte et al. 1993]. However, follow-up studies dealing with the prognostic power of antioxidant status are scarce. In the present study several blood antioxidant parameters previously measured in coal workers are related to prospective changes in pneumoconiosis (chest radiography) and the presence of chronic bronchitis, as well as to retrospective analysis of lung function decline. Aim of this study is to determine the relevance of blood antioxidant status in surveillance and screening of (retired) coal workers. For this purpose discriminant analysis is used to determine to what extent multiple blood antioxidants as indicators of "oxidative stress", may identify coal workers at increased risk for coal dust induced respiratory disorders.

Methods

Study design

Figure 7.1 shows the design of the study. Blood antioxidant parameters, evaluated in red blood cells or in the plasma of 91 coal workers were related to five-year changes in chest radiographs and to 10-year lung function decline. Reduced (GSH) and oxidized (GSSG) glutathione concentrations, total (Gpx-To) and selenium dependent (Gpx-Se) glutathione peroxidase activities, glutathione-

S-transferase activity (GST), superoxide dismutase activity (SOD) and catalase activity were measured in red blood cells and concentrations of Vitamin A, Vitamin E and iron (Fe) were determined in serum. GST levels were measured only in 64 subjects of this cohort [Evelo et al. 1993]. All subjects previously screened for these parameters were recruited for a five-year follow-up and upon written informed consent, a new chest radiograph was made. A questionnaire was combined with a personal interview to determine job-history, smoking and medical status. Furthermore, all medical files were screened retrospective for a 10-year period to determine longitudinal lung function decline (see Figure 7.1).

Figure 7.1. Design of the study.

Antioxidants measured in red blood cells or plasma of coal workers were related to lung function decline over a 10-year interval (retrospective), and to development or progression of CWP, and chronic bronchitis determined at 5-year follow-up (prospective). Lung function decline was determined by longitudinal analysis of spirometry data (FEV1 and FVC) determined between 1987 and 1992. Progression of CWP was evaluated by paired comparison of chest-radiographs (CR) made in 1987 and 1992. Job-history (including calculation of cumulative dust exposure), smoking, medication and medical history (including chronic bronchitis) were determined at follow-up from questionnaires, personal interviews and medical files (see text for details).

Analysis of blood antioxidant parameters

Blood sampling and treatment, and analysis of red cell and plasma antioxidants was done as described previously [Engelen et al. 1990, Evelo et al. 1993]. Briefly, Hb concentration in red blood cells was measured spectrophotometrically. Plasma iron concentrations were determined by the Iron Ferro-Zinc test of Roche Diagnostica [Stookey, 1970]. Antioxidant activities in erythrocytes were determined spectrophotometrically, according to the methods of Maral et al. [1977] (Gpx), McCord and Fridovich [1969] (SOD), Aebi [1984] (Catalase), and Habig and Jacoby [1981] (GST). GSH and GSSG concentrations were determined according to the protocol described by Hissin and Hilf [1976]. Vitamin A and E were simultaneously measured by HPLC according to Cuesta Sanz and Santa-Cruz [1986].

Chest radiography and lung function

Chest radiographs taken in 1987 and in 1992 were scored according to the classification rules of the International Labour Organisation [ILO 1980] by an experienced panel of three physicians. Scoring was done in plenary sessions where consensus had to be reached based on individual scores. Five-year progression or new development of CWP was determined from a paired reading session of each individuals' chest radiograph of 1987 and 1992 [Schins et al. 1994].

Lung function decline (ΔFEV_1 and ΔFVC) was calculated by linear regression for a ten year interval before blood sampling (1978 - 1987). Lung function had been measured periodically at medical screening using a Vitalograph TM spirometer (Vitalograph Ltd, UK) at two mine-pits and a Vicatest 4 (Mijnhardt, Odijk, Holland). Tiffeneau index was calculated from FEV_1 divided by FVC times 100%. Lung function decline of coal miners was calculated as the regression slope obtained with least-square analysis through minimally 8 out of 10 yearly measurements. Lower and upper quartiles of ΔFEV_1 were used as cut off point to define rapid versus slow decliners.

Exposure, smoking, medication, and medical history

Occupational history from each subject was gathered from the personel and medical files. Individual dust exposure was calculated from each individuals' job exposure matrix obtained from a personal interview at follow-up and the medical file. Exposure parameters used for this study were: (I) cumulative dust exposure, (II) cumulative quartz exposure, (III) years underground, and (IV) years underground. Cumulative exposure was calculated as described elsewhere [Schins and Borm., 1995].

A validated questionnaire [Cotes et al. 1987] was sent to all participants in which medical symptoms, medical history, smoking habits and medication were asked. Answers were verified during personal interviews and by comparison with records from the preceding study [Engelen et al. 1990]. Smoking amounts (pack years) were calculated as described elsewhere [Jorna et al. 1994]. The questionnaire was used to identify respiratory symptoms at follow-up. When bronchitis-like symptoms (i.e. presence of both cough and pleghm) were reported for most days of thee week and at least three months per year, the subject was classified as "chronic bronchitis".

Statistical evaluations

Correlations between antioxidant levels and (cumulative) exposure, age, smoking, lung function were evaluated by Spearman Rank correlation. Unpaired differences between 1987 and 1992 (sub) groups were tested by the Mann Whitney U-test. Differences between miners without or with CWP progression, obstruction, and respiratory symptoms were evaluated by the Mann-Whitney test. Finally, stepwise discriminant analysis was used to determine

whether each individuals' "antioxidant status", i.e. the use of multiple antioxidant parameters would allow classification of a coal miners into mutually exclusive groups [Dillon and Goldstein 1984]. Therefore, all subjects were classified in the following groups: Subjects (1) with or without development of CWP, (2) with or without progression of already existing CWP, subjects with (3) slow or rapid longitudinal FEV₁-decline, and (4) subjects with or without chronic bronchitis. Statistical evaluations were made using SPSS 6.1 (Inc. Chicago, IL).

Results

Ultimately 66 out of 91 coal miners previously screened for antioxidant parameters (73% of the original cohort) participated in our follow-up. GST levels were only known for 46 subjects of this group. Lung function decline (retrospective analysis of medical files) was obtained from 40 out of 91 subjects. With regard to these subgroups, no selection bias was observed for age, smoke habits, exposure, medication, pneumoconiotic stage and antioxidants (data not shown). None of the antioxidant levels listed in Table 7.1 was significantly related to individual cumulative (dust/quartz) exposure nor to any other exposure estimate (i.e. years since first exposure or years exposed). Furthermore, no antioxidant levels was significantly related to age or smoking habits (pack years).

Single marker analysis

Based on the paired chest radiograph reading sessions, it was observed that three out of 52 miners newly developed CWP, while 8 out of 14 miners had progression of already existing CWP. None of the initial antioxidant data of these subgroups (Table 7.1) was significantly different for miners with or without disease development or progression, only red cell SOD activity was significantly higher in miners with subsequent progression of CWP ($p < 0.05$). As can be seen in Table 7.1, this finding was not biased by differences in distributions of age, smoking or (cumulative) exposure. In the miners without development of CWP, one subject was identified with red blood cell catalase deficiency. Statistical analysis was repeated upon exclusion of this subject (see legend of Table 7.1).

The average decline in FEV₁ was -34.1 mL/year ($n=40$), and based on upper and lower quartiles subjects were divided into rapid (cut off -68.4 mL/year) or slow decliners (cut off -6.9 mL/year). However, no significant correlations were observed between longitudinal lung function decline (Δ FEV₁ or Δ FVC) and individual antioxidant parameters, nor was any significant difference seen between slow and rapid decliners for any antioxidant (see Table 7.2). FEV₁-decline was not significantly different between subjects with or without progression of pneumoconiosis (see Table 7.1).

Table 7.1 Blood antioxidant parameters, age, exposure, smoking and the 5-year progression of pneumoconiosis in the coal workers.

	Reference miners		Miners with CWP	
	NP (n=49)	P (n=3)	NP (n=6)	P (n=8)
Age, years (in 1992)	47.6 (0.7)	45.3 (3.8)	48.6 (1.4)	52.6 (1.3)
Underground in 1987, years	21.8 (0.6)	23.0 (4.6)	24.2 (1.1)	26.0 (1.1)
Total underground (1992), years	22.9 (0.6)	24.0 (4.0)	25.0 (1.4)	26.6 (1.4)
Cumulative dust, gh/m ³ (in 1992)	95 (8)	135 (33)	141 (4)	123 (25)
Smoking, packyears (in 1992)	124 (17)	143 (34)	198 (71)	190 (45)
FEV ₁ -decline (mL/year) ^a	-30.1 (9.9)	-69.8 (43.2)	-36.1 (14.3)	-82.1 (41.5)
Chronic bronchitis, yes / no ^b	8 / 41	1 / 2	1 / 5	2 / 6
Hb, mg Hb/mL	15.1 (0.28)	13.9 (0.33)	14.4 (0.31)	15.8 (0.46)
SOD, U/gHb	1979 (74)	1740 (190)	1703 (156)	2308 (155)*
Catalase, μ mole H ₂ O ₂ /min/g Hb ^c	0.45 (0.01)	0.50 (0.04)	0.43 (0.02)	0.46 (0.02)
GSHpx-To, μ mole NADPH/min/g Hb	9.67 (0.09)	9.79 (0.15)	9.64 (0.20)	9.77 (0.13)
GSHpx-Se, μ mole NADPH/min/g Hb	9.25 (0.11)	9.39 (0.28)	9.20 (0.33)	9.39 (0.16)
GST, U/gHb ^d	3.11 (0.23)	2.35 (0.50)	3.63 (0.42)	2.54 (0.34)
GSH, mmole/g Hb	3.99 (0.09)	3.38 (0.42)	3.92 (0.25)	3.84 (0.20)
GSSG, μ mole/g Hb	.036 (.001)	.033 (.004)	.037 (.003)	.035 (.002)
Fe, μ mole/mL plasma	18.3 (1.1)	21.0 (5.8)	18.5 (1.9)	14.7 (1.3)
Vitamin A, μ g/mL plasma	9.4 (0.4)	7.9 (0.6)	11.2 (1.3)	8.9 (0.9)
Vitamin E, μ g/mL plasma	14.6 (0.7)	11.7 (2.8)	20.9 (4.3)	14.0 (1.7)

P, progression (or development) of pneumoconiosis; NP, no progression of CWP; The asterisk (*) denotes a significant difference from NP, $p < 0.05$, Mann-Whitney U-test.

^a FEV₁-decline was obtained from 35 miners (i.e. n= 26, n=2, n=4, n=3 subjects respectively); ^b Odds ratios for chronic bronchitis and progression were 2.6 in references and 1.7 in CWP group, both not significant at 90 % C.I.; ^c Upon exclusion of one catalase deficient subject, average of NP references becomes 0.46 (0.01); ^d GST was related to progression in 46 miners (i.e. n= 33, n=2, n=6, n=5 subjects respectively).

Based on the questionnaires, 18 and 12 subjects were classified having bronchitis or chronic bronchitis at follow-up. Both development or progression of CWP were independent from the presence of (chronic) bronchitis (see Table 7.1), and FEV₁-decline was not different between subjects with or without chronic bronchitis (see Table 7.2). Interestingly, in subjects with chronic bronchitis red cell GSH concentration was significantly reduced compared to normals ($p < 0.01$).

Table 7.2 Blood antioxidant parameters, age, exposure, smoking and the 10-year retrospective FEV1-decline in the coal workers.

	FEV1-decline		Chronic bronchitis	
	high (n=10)	low (n=10)	Yes (n=12)	No (n=53)
Age, years (in 1992)	48.2 (1.7)	46.1 (1.2)	49.5 (1.6)	47.9 (0.6)
Underground in 1987, years	23.0 (3.9)	22.1 (1.3)	23.2 (1.3)	22.5 (0.6)
Total underground (1992), years	23.8 (1.0)	23.5 (1.4)	23.8 (1.3)	23.6 (0.6)
Cumulative dust, gh/m ³ (in 1992)	100 (19)	93 (18)	89 (17)	108 (8)
Smoking, packyears (in 1992)	199 (51)	136 (35)	161 (38)	135 (17)
FEV1-decline (mL/year) ^a	-100.4 (9.5)	17.0 (10.0)**	-10.1 (29.3)	-44.6 (7.8)
Chronic bronchitis, yes / no ^b	1 / 9	2 / 8		
Hb, mg Hb/mL	15.8 (0.6)	14.7 (0.6)	15.2 (0.5)	15.0 (0.2)
SOD, U/gHb	2079 (287)	1989 (128)	2171 (126)	1941 (71)
Catalase, μ mole H ₂ O ₂ /min/g Hb	0.46 (0.02)	0.43 (0.01)	0.43 (0.01)	0.47 (0.01)
GSHpx-To, μ mole NADPH/min/g Hb	9.86 (0.12)	9.48 (0.29)	9.78 (0.16)	9.65 (0.08)
GSHpx-Se, μ mole NADPH/min/g Hb	9.35 (0.13)	9.22 (0.27)	9.36 (0.21)	9.24 (0.10)
GST, U/gHb ^c	3.09 (0.27)	2.87 (0.57)	3.32 (0.27)	2.99 (0.21)
GSH, mmole/g Hb	4.00 (0.18)	4.12 (0.15)	3.53 (0.16)	4.05 (0.09)**
GSSG, μ mole/g Hb	.035 (.003)	.038 (.003)	0.035 (.003)	0.035 (.001)
Fe, μ mole/mL plasma	16.9 (1.3)	18.1 (2.0)	18.7 (3.5)	18.0 (1.0)
Vitamin A, μ g/mL plasma	10.5 (1.3)	10.6 (1.2)	10.6 (0.9)	9.2 (0.4)
Vitamin E, μ g/mL plasma	16.6 (2.3)	16.7 (1.8)	17.7 (1.7)	14.5 (0.8)

** p < 0.01 (Mann-Whitney U-test); The catalase deficient subject was excluded from analysis.

^a FEV1-decline determined in n= 10, n=10, n=5, n=35 subjects respectively; ^b Odds ratio= 0.44, for chronic bronchitis vs. lung function decline; not significant at 90 % C.I.; ^c GST subgroup numbers are 7 vs 9, and 7 vs 38 respectively.

Multiple marker analysis

To determine the involvement of multiple antioxidants in specific disease, a discriminant analysis was performed. Due to the distribution skewness of several antioxidant parameters, analysis was performed on logarithm-transformed data. Antioxidants tested in the discriminant function were: Catalase, SOD, GSH (i.e. reduced glutathione only), GST, Gpx (i.e. total), Fe, Vitamin A and Vitamin E. Missing values (for GST, Vitamin A and Vitamin E) were replaced with the (ln-transformed) group average. Furthermore, a catalase "outlier" subject was excluded from all multiple statistical analyses. The results of the stepwise

analysis are shown in Table 7.3, for several discriminating criteria along with the percentage of correct grouped subjects and its significance (χ^2), and the antioxidants significantly attributing to the correct grouping. 86% of the subjects were correctly grouped into progression (n=8) or no progression (n=6) of already existing CWP, when erythrocyte GSH concentration, SOD and GST activities, and serum Vitamin E status were included in the discriminant function (df=4, χ^2 =14.2, $p<0.01$). The discriminant function indicated a negative association with disease progression for GSH, Vitamin E and GST, while SOD was higher in those with progression. Furthermore, a significant discrimination of subjects classified according to new development of CWP (3 versus 48 subjects) was observed with GSH, Vitamin (both negative) and catalase and iron (positive) in the discriminant model. A discrimination between slow and rapid decliners in FEV₁ was obtained with SOD, catalase, glutathione-S-transferase and glutathione peroxidase in the model. Interestingly, these parameters were all increased in those with rapid decline in FEV₁ in comparison with those with a low decline. The presence or absence of chronic bronchitis was significantly discriminated with GSH, SOD and Vitamin A, i.e. explained by lower GSH and increased SOD and Vitamin A levels in the symptomatic group.

Table 7.3 Discriminant analysis of antioxidants in erythrocytes and plasma in relation to coal dust induced respiratory disorders

Classification criterion	yes/no	Grouped (%)	χ^2	df	p-value	Variables included in discriminant model
Development of CWP	3 / 48	86.0	12.3	4	<0.02	GSH (-), VitE (-), Fe (+), Cat (+)
Progression of CWP	8 / 6	85.7	14.2	4	<0.01	GSH (-), VitE (-), GST (-), SOD (+)
Lung function decline	10 / 10	85.7	10.2	4	<0.04	Gpx (+), SOD (+), GST (+), Cat (+)
Chronic bronchitis	12 / 53	70.3	12.6	3	<0.01	GSH (-), SOD (+), VitA (+)

All subjects were classified as follows: (1) presence or absence of development of CWP; (2) presence or absence progression of already existing CWP; (3) rapid (= "yes") or slow (= "no") decline in lung function (i.e lower versus upper quartile of Δ FEV₁).

Stepwise discriminant analysis was performed on natural logarithm-transformed data. Antioxidants tested in the discriminant function were: Catalase, SOD, GSH, GST, Gpx (total), Fe, Vitamin A and Vitamin E. Missing values were replaced with the group average. For each classification factor, the Table shows the percentage of correct grouped subjects, the significance of the canonical correlation and the antioxidants significantly attributing to the correct grouping, i.e. positive (+) or negative (-) in the canonical correlation function.

Discussion

As stated by Halliwell and Cross [1994], the major issue is not whether oxidative stress can be demonstrated in a disease, but whether it makes a significant contribution to the disease activity. Previously, we and others elucidated the first question for subjects chronically exposed to coal dust by showing different antioxidant status in stages of coal workers pneumoconiosis [Engelen et al. 1990, Evelo et al. 1993, Perrin-Nadif et al. 1996]. Glutathione concentrations as well as glutathione-S-transferase activities were significantly decreased at early stage pneumoconiosis, while at later stages erythrocyte GSH and GST were back to normal [Engelen et al. 1990, Evelo et al. 1993]. In these cross-sectional studies, our original hypothesis was that subjects who develop pneumoconiosis are "less well equipped to deal with reactive oxygen species" [Engelen et al. 1990], and that erythrocytes could be considered as important circulating antioxidant carriers in this disease. Further support for this hypothesis is provided by our recent observation that oxidative DNA damage (i.e. 8-hydroxydeoxyguanosine residues) is increased in peripheral blood lymphocytes of coal workers compared to non-exposed controls [Schins et al. 1995]. However, whether the variation in oxidative stress observed in coal workers significantly attributes to respiratory disease, would necessitate a different experimental approach.

In the present study, prospective analysis of the above cohort since 1987 has shown that red blood cell SOD activity was significantly higher in miners with subsequent progression of pneumoconiosis as compared to those without progression at five-year follow-up. Previously we reported that SOD activities were not increased in coal workers with pneumoconiosis compared to healthy miners [Engelen et al. 1990]. However, increased erythrocyte SOD levels were found recently in underground coal miners compared to surface workers [Perrin-Nadif et al. 1996]. This might be a consequence of differences in coal dust exposures but one could also consider these findings to reflect the higher pneumoconiosis risk for underground versus surface workers. Previously, it has been shown that macrophage superoxide anion ($O_2^{\bullet-}$) generation was increased in coal workers compared to non-exposed controls [Rom et al. 1987, Wallaert et al. 1990], and the activity of SOD may therefore reflect the extent of a respiratory $O_2^{\bullet-}$ burst from macrophages and neutrophils in the lung. This is underscored by animal data, demonstrating a direct upregulation of manganese SOD expression and immunoreactive protein in the lung by mineral dusts, which was related to the inflammatory response [Janssen et al. 1992].

Apart from SOD, also other antioxidants were consistently different between subgroups although sometimes only on borderline significance. A combination of antioxidant parameters tested by discriminant analysis, showed that in addition to SOD, also GST, GSH and Vitamin E status denominated presence or absence of CWP progression. However, in contrast to SOD activity, these

parameters were reduced in those with progression (see also Table 7.1). Interestingly, Vitamin E and GSH were also reduced in the few (n=3) healthy miners who developed pneumoconiosis during follow-up, while in those subjects catalase activity and plasma iron were increased. Therefore, reduced levels of red cell glutathione and plasma Vitamin E status may be considered as risk factors for CWP. The concept of reduced GSH as a possible fibrotic risk factor, was also discussed by Cantin et al. who showed that reduced extracellular GSH was associated to increased lung fibroblast proliferation [Cantin et al. 1990]. The significance of reduced GST in fibrosis may be closely related to this observation. Its reduced activity may be related to oxidative stress during lipid peroxidation [Halliwell and Cross 1994], which is considered as an important feature of mineral dust related lung toxicity. Another support for the involvement of GST in fibrosis comes from Smith et al. [1994], who recently showed that GST- μ class (GST-M1) deficient subjects were at increased risk for asbestosis. Although the majority of GST activity in erythrocytes originates from GST-P class isoenzymes, GST polymorphism could, in addition to its role in (smoking related) lung cancer [Seidegard et al. 1986, 1990], be significantly involved in the development of pneumoconiosis. Vitamin E in those at risk may be decreased because of its importance as chain reaction terminator in the lipid peroxidation process. Interestingly, erythrocyte GSH levels were not only associated with increased pneumoconiotic risk, but also dramatically reduced in subjects with chronic bronchitis symptoms reported at follow-up. Remarkably, all "enzymatic" antioxidants (GST, Gpx, SOD and catalase) were increased in those with rapid decline in FEV₁ versus those with a slow decline. Since longitudinal decline was analysed retrospectively, our observations could be explained as a compensatory upregulation of enzymatic antioxidants in those with reduced "nonenzymatic" antioxidant status. Reduced intake of Vitamin A [Morabia et al. 1989], as well as Vitamin C and E [Morabia et al. 1989, Schwartz and Weiss 1994, Britton et al. 1995] is associated with increased risk of progressive lung function loss and airway obstruction. Others have shown reduced GSH levels in bronchoalveolar lavage fluid in subject with obstructive lung disease [Linden et al. 1995].

Finally, this study enabled us to test the actual involvement of oxidative stress in coal dust induced respiratory disorders. We found that in CWP reduced erythrocyte GSH and Vitamin E content were risk factors while increased activities of catalase and SOD in red cells tended to be risk factors. At the same time, an over-all increase in enzymatic antioxidant capacity identified subjects, with a rapid retrospective lung function decline. GST activity showed a contrasting discriminative power; those with lower GST activity were at increased risk for progression of coal workers pneumoconiosis; however a lower lung function decline was observed in this group. Underscored by recent findings (GST polymorphism) we believe that this finding deserves further investigation. Although pneumoconiotic and nonpneumoconiotic endpoints were

independent, it can not be ruled out that both effects may be closely related to each other. On the one hand, a reduced intake of antioxidants -associated to lung function loss- may also be a risk factor for coal dust induced pulmonary fibrosis. On the other hand, coal dust exposure and its associated generation of ROS could also play a role in some of the nonpneumoconiotic effects such as chronic bronchitis or chronic lung obstruction. As such, measurement of multiple antioxidants in the peripheral blood may be relevant for surveillance and screening of (retired) coal workers. To our opinion, these observations should be extrapolated to other cohorts of coal (or other mineral) dust exposed subjects, to validate whether antioxidant status patterns of the peripheral blood as reported above can be considered as useful biological markers in subjects chronically exposed to coal (i.e. mineral) dust.

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PART 2

Cytokines

Chapter 8

EPIDEMIOLOGICAL EVALUATION OF MONOCYTE TNF- α RELEASE AS AN EXPOSURE AND EFFECT MARKER IN PNEUMONOCOINOSIS. A FIVE YEAR FOLLOW UP STUDY AMONG COAL WORKERS.

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Abstract

Objectives - To determine (a) reproducibility with previous cross-sectional findings, and b) the predictive value of initial release of tumour necrosis factor- α (TNF- α) towards later progression of coalworkers' pneumoconiosis CWP.

Methods - Release of monocyte TNF- α after in vitro stimulation with coal mine dust, silica, and endotoxin was measured in 104 retired miners and was related to stage of CWP (chest radiograph) and cumulative exposure. A subgroup of 46 miners was screened by high resolution computed tomography (HRCT). Prospective analysis of TNF- α (40 out of 104 miners involved in the previous TNF- α study) was done by relating initial TNF- α to five year progression of CWP, measured by comparison of paired chest radiographs.

Results - As observed previously, dust stimulated release of TNF- α was increased in miners, especially in the early stages of pneumoconiosis. Cumulative exposure was related to pneumoconiotic stage but not to release of TNF- α . This excluded TNF- α as a exposure marker. Initial concentrations (1987) of TNF- α were related to later progression of CWP. Miners who showed abnormally high dust stimulated release of TNF- α had an increased risk of progression in CWP (relative risk 8.1).

Conclusions - These results show (a) the significant involvement of TNF- α in pneumoconiosis in humans induced by coal dust and (b) that this routine test possibly constitutes a powerful tool to estimate individual prognosis of pneumoconiotic disease, even after the end of occupational exposure.

Introduction

Pulmonary fibrosis should be looked upon as a pathological process dependent upon many cell types, in which alveolar macrophages, lung epithelium, fibroblasts, type II cells, PMN, and many other cells are involved [Elias et al. 1990; Crystal et al., 1991]. These cells are known to produce various factors (reactive oxygen species, cytokines, enzymes, etc.) that are thought to be crucial mediators of the hallmarks of pulmonary fibrosis: excessive fibroblast proliferation and collagen deposition [Crystal et al., 1991; Snyder, 1988]. Mediators that have been put forward, mostly from animal studies, are: Platelet derived growth factor- β (PDGF- β), Tumor necrosis factor- α (TNF), Interleukin- 1β and Interleukin-6 (IL- 1β , IL-6) and prostaglandin-E₂ (PGE₂) in asbestosis [Brody 1993; Perkins et al., 1993] or TNF, IL-1 and the Macrophage inflammatory proteins 1 α and 2 (MIP-1 α , MIP-2) in silicosis [Piguet et al., 1990; Driscoll et al., 1990; Mohr et al., 1991; Driscoll et al., 1993]. The potential of many of these cell mediators as markers of exposure, disease susceptibility or effects has been raised many times, but studies on the relations between exposure, disease, and cytokine release in occupationally or environmentally exposed subjects are scarce.

In 1987 several case-control studies among coal miners were performed in our laboratory to look for a marker to explain disease among exposed miners with and without coal workers' pneumoconiosis (CWP). The serum concentration of procollagen type III peptide was measured to assess fibrotic activity versus pneumoconiotic stage [Janssen et al., 1992]. Another study focused on the relation between CWP and antioxidant expression [Engelen et al., 1990]. The *in vitro* secretion of TNF by peripheral blood monocytes [Borm et al., 1988] of healthy miners and miners with CWP, matched on age and underground working years, showed that the release of TNF in monocytes could be stimulated *in vitro* by coal mine dust, and that (healthy) coal miners had a higher release of monocyte TNF upon *in vitro* stimulation than did controls who were not exposed to dust. Moreover, a decreasing trend in the release of TNF was seen along with the clinical progression of CWP possibly as a positive feedback mechanism to prevent concentration of fibrosis, and in healthy miners (0/0) and miners with early stages of CWP (0/1) a large variation in the release of TNF was found. We then suggested that people who showed values largely exceeding the normal range of controls who were not exposed to dust, were at risk of development of CWP and concluded that 'definitive proof for the role of mononuclear phagocyte-derived TNF in the pathogenesis of CWP and other fibrotic lung diseases has to originate from laboratory animal studies and prospective epidemiologic surveys among those at risk' [Borm et al., 1988]. Several years ago, Piguet *et al.* [1990] showed the crucial role of TNF fibrosis induced by silica with a mouse *in vivo* model. Since then other studies have firmly established the importance of TNF in lung fibrosis induced by silica

[Driscoll et al., 1990; Mohr et al., 1991; Phan et al., 1992]. Also *in vitro* studies have emphasised the involvement of TNF in the complex cytokine network of lung tissue [Elias et al., 1990; Perkins et al., 1990; Warren et al., 1988; Everson and Chandler, 1992]. Lasalle and coworkers reported an increased spontaneous release of TNF by alveolar macrophages of active healthy coal miners and miners with CWP compared with controls [Lasalle et al., 1990]. The importance of these findings for the prevention of lung fibrosis induced by mineral dust remains an open question. In 1992 all miners involved in our cross sectional studies were recruited for a five year follow up to investigate whether our previous cross sectional findings on release of TNF in subjects exposed to dust and disease stage of CWP could be validated, and to determine the relative risk of development of CWP for coal miners who showed *in vitro* release of TNF.

Methods

STUDY GROUP

Miners (n=156, fig 1) from the Belgian coal mining industry "Kempense Steenkoolmijnen" involved in previous studies in 1987 [Borm et al., 1988; Engelen et al., 1990; Janssen et al., 1992] were asked to participate in the follow up study that included a chest radiograph, several lung function tests, medical and exposure history, and the blood variables TNF, PIIP and red blood cell antioxidant enzymes (AOE). Chest radiographs taken in 1987 and in 1992 were scored according to International Labour Organisation classification rules [ILO, 1980] by an experienced panel of three physicians. A subgroup of 46 miners was screened by high resolution computed tomography (HRCT) by invited participation. Percentages of cases and control miners in the HRCT subgroup (i.e. 13 v 23) were equal to those of the large cohort (i.e. 76 v 28), and none of the variable; age, smoking habits or exposure in the subgroup were different from the large cohort (n=104). A control group of 44 age matched men participated in the study on voluntary basis. Exclusion criteria for controls not exposed to dust were: working for more than one year in an occupational setting with chronic dust exposure (mining, cement works, ceramic industry, bakery, etc.) and one or more of: asthma, (chronic) bronchitis, and other chronic diseases of lung, liver and kidney. Based on these criteria, 29 controls were included in statistical analysis.

TNF-assay

Release of monocyte TNF was measured as described previously [Borm et al., 1988]. Briefly, blood (3 x 10 ml) was sampled and left overnight in tubes with EDTA at room temperature. White blood cells were isolated by buoyant density centrifugation (Lymphoprep-Nycomed), adjusted to concentrations of 0.5×10^7

cells/ml and seeded (0.5 ml) to tissue culture dishes (Costar TC-24) to allow adherence (repetitive incubation, agitation and washing). Adherent monocytes were stimulated with coalmine dust (5 mg/ml), silica particles (0.5 mg/ml) or with *Escherichia coli* 0111B4 LPS endotoxin (3 and 1000 ng/ml). Silica and endotoxin were obtained from Sigma (St Louis USA); the coal mine dust (sampled from a coal pit) was obtained from the same batch as used previously [Borm et al., 1988]. Each person's baseline release of TNF was measured without addition of any *in vitro* stimulant. After 18 hours of incubation (37°C, CO₂) the cell free supernatants were stored at -30°C until analysis by a specific sandwich ELISA for TNF as described previously [Borm et al., 1988; Debets et al., 1988].

QUESTIONNAIRE

An extensive questionnaire was sent to all participants in which medical complaints, medical history, smoking habits, and medication were asked for. Answers were all verified during personal interviews at the time of medical screening and by comparison with questionnaires from the data records of our preceding studies. Amount of smoking was expressed as: packets of cigarettes/week x years (pack-years) smoked. One packet was considered to consist of 25 cigarettes; the smoking of one cigar was considered to be equivalent to 3 cigarettes; and 50 g (pipe-) tobacco to be equivalent to 75 cigarettes. Furthermore all subjects were subdivided into current, former, and never smokers.

Subjects were classified for medication and medical history based on the presence or absence of any medication during three weeks before blood sampling. Disease was determined in two ways; the presence or absence of any medical complaint for at least three days, and of specific disease of the lung, kidney, liver, or any other chronic disease for at least three years before blood sampling.

EXPOSURE

Much emphasis was put on a good estimation of exposure and several variables were used: (a) time worked underground, (b) time since first occupational exposure, (c) time since last occupational exposure, (d) cumulative exposure to respirable coal dust and quartz.

Years exposed , first exposure, and last exposure

Years exposed, time since first exposure and time elapsed since last occupational exposure were determined from the medical file and a personal interview at follow up. Exposure years were determined from the complete job history obtained from each subject and defined as the total number of years in which a subject worked underground in a coal mine. Long term absenteeism -for example, for disease or through special leave- as well as interim job changes were taken into account. Years since first exposure were defined as the interval between the year in which a miner started working underground and the time of

our study. Time from last exposure was the interval between retirement and our follow up in months.

Calculation of cumulative dust exposure

Occupation history, gathered from the medical file and verified by a personal interview, was used to calculate exposure for all periods worked at and out of the coalfaces. The cumulative dust exposure was calculated by summation of the products of the yearly mean dust concentrations for the colliery where the miner was appointed with the average time worked underground during that year.

The formula was:

$$E_x = \sum_{i=1}^n (C_i \cdot T_x) \quad , (\text{ghm}^{-3}) \quad (1)$$

where, E_x = cumulative dust exposure (ghm^{-3}) of miner x ; C_i = mean dust concentration ($\text{g} \cdot \text{m}^{-3}$) in year i ; T_x = time worked underground (h) by miner x ; n = years of employment.

The resulting individual exposure is expressed in ghm^{-3} of sampled air. The yearly mean dust concentrations (C_i) were derived from dust measurements regularly made to check compliance with exposure limits in every coal face of the colliery. Since 1965 these measurements have been carried out with a STASER sampler (jointly developed by a Belgian manufacturer and the Dutch State Mines) at fixed points, 15 m from the face line, in the return end gate. The concentrations before 1965 have been estimated on the basis of preliminary studies carried out in preparation of the implementation of dust regulations. The total dust concentrations have been converted into "respirable" dust concentrations by the formula:

$$rd = 1.1 \sqrt{td} \quad , (\text{ghm}^{-3}) \quad (2)$$

where, rd = respirable dust concentration (gm^{-3}); td = total dust concentration (gm^{-3}), as measured.

This empirical relation (equation 2) was derived from experimental side-by-side instrument comparisons conducted in 1984-5 with the French CPM3 instrument and the STASER sampler under various mining conditions in the Belgian coal mines (144 paired measurements). The time worked underground was estimated for each subject assuming that on average 1000 hours a year were worked underground : six hours at work a shift (allowance being made for travelling

time) $\times$ 220 shifts a year $\times$ (100-25)/100 (where 25% is taken as a rate of absenteeism). The exposure outside the coal face was estimated on the basis of professional judgement of the "dustiness" of the occupation and the knowledge of normal respirable dust levels in that job (non-mechanised and mechanised headings, stone drivages, etc). The cumulative quartz exposure (mghm-3) has been estimated, based on the average quartz content of Belgian coalface (5%) and stone drive dust (10%).

RADIOGRAPHY

Protocol and classification

Chest radiographs (46 $\times$ 46 cm) were taken posterior to anterior at maximal inspiration (1.5 m distance, 70 kV) and scored according to the ILO criteria [ILO, 1980]. Scoring was done in plenary sessions where consensus had to be reached based on individual scores. The reading panel consisted of three occupational physicians of whom two were seeing more than 3000 chest radiographs a year, and one is a trained reader but had not been active as such for the last three years. Two readers in our follow up study had been involved with the original study. Standard ILO chest radiographs were used as reference to classification. Based on the ILO scores the cohort was subdivided into five groups: ILO classification 0/0 (group 0), class 0/1 (group 1), classes 1/0, 1/1 and 1/2 (group 2), classes 2/1, 2/2 and 2/3 (group 3) classes 3/2 and 3/3 (group 4), and finally , and 3/+ and subjects with progressive massive fibrosis (group 5). Furthermore miners were grouped as either "healthy coal workers" with ILO classification of 0/0 (group 0 only) or as miners with evidence for CWP with ILO classification of $\geq$ 0/1 (groups 1 to 5). Five year changes in pneumoconiosis were determined by comparison of each man's chest radiographs of 1987 and 1992. All radiographs were scored in a randomised blind reading session, as well as in pairs; the paired reading session was used as definitive evidence of progression as this direct comparison method allows the best validation of TNF as marker of five year changes in CWP.

High Resolution Computed Tomography

The HRCT scans were made (Somatom plus, Siemens, Erlangen, Germany) and visually scored by two radiologists as described elsewhere [Lamers et al., 1994] in a subgroup of 46 miners. Briefly, four HRCT slices, two in the upper lobes (3 and 5 cm above the carina) and two in the lower lobes (3 and 5 cm below the carina) were obtained at full inspiration in a supine position, and scored independently by two thoracic radiologists without knowledge of clinical findings. Each slice was visually assessed for the presence of non-calcified parenchymal opacities with almost the same basic principles as the ILO system for grading pulmonary disease on chest radiographs. The profusion score was converted to a linear scale that ranged from zero to 10 as follows: "0/0 = 0, 0/1 = 1, 1/0 = 2, 1/1 = 3, 1/2 = 4, 2/1 = 5, 2/2 = 6, 2/3 = 7, 3/2 = 8, 3/3 = 9 and 3/+ = 10" [Lamers et al., 1994; Rémi-

Jardin et al., 1990]. The score of the eight lung fields was summed up and a total score was calculated. The mean score of both observers (correlation 0.86, $p < 0.001$) was calculated for the purpose of this study. Our attention was mainly focused on the relation between TNF and HRCT lesions in a group of 35 miners without the classical diagnostic evidence for CWP (ILO $\leq 0/1$).

DATA EVALUATION AND STATISTICAL ANALYSIS

Figure 8.1 schematically shows the composition of the study group. Exploratory analysis was performed on the 1992 cross-sectional data, to study the confounding effects of age, smoking, medication, and variables of exposure. Reproducibility with cross sectional data of 1987 was then evaluated by unpaired statistical analysis of data obtained in 1987 [Borm et al., 1988] *v* data obtained in 1992 and by cross sectional analysis of 1992 data as previously described for the 1987 cross sectional data [Borm et al., 1988]. Longitudinal study analysis was performed by paired statistical analysis of 1987 and 1992 data and predictive values of variables of 1987 (prospective analysis) or 1992 (retrospective analysis) were related to five year changes in radiological severity of CWP.

Figure 8.1. Construction of the follow up study among retired coal miners previously (1987) involved in cross-sectional studies on the role of TNF- α (n=66) or antioxidants (n=90) in coal workers pneumoconiosis. The shaded area represents the groups in which TNF- α was determined. Data interpretation is done either by comparison of the TNF- α follow up group (n=40) (longitudinal analysis) or of the total 1992 cohort (n=104) with the TNF- α group in 1987 (n=66) (cross sectional analysis). Chest radiographs were taken for all miners in 1987 (n=156), and for all miners involved in 1992 (n=104), allowing 104 paired chest radiograph comparisons (determination of five year progression of CWP). From the 1992 cohort (n=104), 46 miners were screened by high resolution computed tomography by invited participation.

Correlations between release of TNF and exposure, age, smoking were evaluated by multiple linear regression. Differences between miners without or with CWP and non-exposed controls, and effects of medication, medical history, and smoking were tested by the Kruskal Wallis test. Trend analysis between pneumoconiotic stage (chest radiographs, HRCT) and TNF, exposure, or other variables was analysed by Spearman's rank correlation. For this purpose, profusion scores were converted to a scale from 0 to 11 for chest radiograph classification and 0 to 80 for HRCT as already indicated. Positive predictive value of five year changes in chest radiograph, sensitivity, and specificity of initial release of TNF were determined. The relative risk of progression of CWP based on 1987 TNF data was determined with a cut off point of 2 SDs above the mean release of TNF measured in the non-exposed control group of 1987 [Borm et al., 1988]; Similarly, the odds ratio of cumulative dust exposure with progression of CWP was determined retrospectively, with the mean cumulative exposure of the 1992 cohort as a cut off point. Also, to determine the significance of our findings at various cut off points, receiver operating characteristics (ROC) curves were calculated [Hanley and McNeil., 1982; van der Schouw et al., 1993]. Statistical evaluations were made with STATGRAPHICS/6.0 (Manugistics, Rockville MD).

Results

ANALYSIS OF EXPLORATORY DATA

Age, smoking, medication and exposure

Table 8.1 shows group characteristics of the total study cohort of 1992. The average age of the control group not exposed to dust was significantly higher than the control miners. No correlation was observed between age and release of TNF in any of the groups (Table 8.2). Smoking habits did not differ among the three groups (Table 8.1), but release of monocyte TNF upon stimulation with coal dust was significantly inversely related to packyears in the miners (n=104, Table 8.2). The dust stimulated release of TNF was significantly lower in ever smokers (1.68 ng/ml, n=61) *v* never smokers (2.90 ng/ml, n=15) (ANOVA, $p<0.01$) but not significantly different between active smokers (n=27) and non-smokers (n=49). Furthermore, smoking was related to release of TNF upon stimulation with coal dust and low concentration of endotoxin (3 ng/ml) in miners without CWP, and to high concentration of endotoxin (1000 ng/ml) in the controls not exposed to dust. In the controls not exposed to dust the average release of TNF upon stimulation with 1000 ng/ml endotoxin was significantly different for active smokers (1.63 ng/ml, n=16) *v* non-smokers (3.16 ng/ml, n=13), and for (ever) smokers (1.85 ng/ml, n=22) *v* never smokers (3.76 ng/ml, n=7) (ANOVA, $p<0.05$). Differences in release of TNF with respect to medication were not observed in any group. Miners with CWP had significantly higher

cumulative exposure and underground working years than the control miners (Table 8.1). No significant relation was found between any of the exposure variables and release of TNF (Table 8.2).

Table 8.1 Group characteristics of the study cohort in 1992 (n=133).

	Controls not exposed to dust (n=29)	Controls healthy miners (n=76)	Miners with CWP [†] (n=28)
Age (years)	50.3 (1.0)*	47.7 (0.6)	49.9 (1.1)
Smoking (packyears)	115 (20)	111 (12)	150 (24)
Smoking (yes/no)	16/13	28/48	14/14 [†]
Smoking (ever/never)	22/7	62/14	23/5
Medication (yes/no)	8/21	20/56	7/21
Cumulative exposure:			
dust (gh/m ³)	-	91.1 (6.4)	130.9 (9.3)**
quartz (mgh/m ³)	-	4798 (306)	6751 (461)**
Years underground	-	22.2 (0.4)	25.0 (0.9)**
Years since first exposure	-	28.9 (0.6)	30.7 (1.0)
Months retired	-	46.3 (2.1)	53.9 (2.1)

The asterisks denote a significant difference towards the control miners group: * $p < 0.05$ ** $p < 0.01$ (Mann-Whitney); Values are mean (SEM); [†] Proportion of smokers/former smokers/never smokers in the ILO profusion categories was: 4/2/1 (0/1); 1/2/2 (1/0, 1/1, 1/2); 3/2/2 (2/1, 2/2, 2/3); 4/2/0 (3/2, 3/3); and 2/1/0 (3/+, PMF)

REPRODUCIBILITY OF CROSS-SECTIONAL DATA

Reproducibility of TNF data

Figure 8.2 shows the monocyte release of TNF for both cross-sectional studies in 1987 and 1992, for the controls not exposed to dust, the control miners, and miners with pneumoconiosis. Unpaired *t* tests of all miners involved in the original study and in the entire follow up group showed that both the spontaneous release of TNF and the release of TNF upon stimulation with low endotoxin (3 ng/ml) in 1992 (n=104) were both significantly lower than in 1987 (n=66); conversely, release of TNF stimulated with coal mine dust was significantly higher in 1992 than in the 1987 study ($p < 0.05$). Spontaneous release of TNF and release of TNF upon stimulation with low endotoxin were significantly reduced in the healthy coal miners of 1992 (n=76) *v* 1987 (n=39) as well as in the miners with CWP of 1992 (n=28) *v* 1987 (n=27). On the other hand, stimulation with coal mine dust released significantly higher concentrations of TNF in the healthy miners of 1992 *v* 1987, but not in the miners with CWP of

1992 *v* 1987. Release of TNF upon dust stimulation was also significantly increased in the controls not exposed to dust in 1992 (n=29) *v* the control group of 1987 (n=12). Other (unpaired) differences were not observed.

Table 8.2 Linear correlations between TNF and exposure, age, and smoking

ILO classification		Release of TNF by stimulation with:				
		Spontaneous	Endotoxin 3 ng/ml	Endotoxin 1000 ng/ml	Coal dust 5 mg/ml	Silica 0.5 mg/ml
<i>cumulative dust (ghm⁻³)</i>						
0/0	(76)	0.01	0.17	0.02	0.00	0.10
≥0/1	(28)	-0.28	-0.10	-0.02	-0.20	-0.06
Total	(104)	-0.06	0.08	0.01	-0.04	0.03
<i>cum. quartz (mghm⁻³)</i>						
0/0	(76)	0.03	0.19	0.02	0.05	0.11
≥0/1	(28)	-0.33	-0.05	0.06	-0.23	-0.10
Total	(104)	-0.06	0.11	0.03	-0.02	0.03
<i>years underground</i>						
0/0	(76)	-0.05	0.12	-0.03	-0.02	0.00
≥0/1	(28)	0.05	0.16	0.30	0.10	0.16
Total	(104)	-0.04	0.12	0.08	0.02	0.05
<i>years since first exposure</i>						
0/0	(76)	-0.22	0.01	-0.06	-0.17	-0.18
≥0/1	(28)	0.08	-0.01	0.16	-0.02	0.00
Total	(104)	-0.15	0.00	0.01	-0.12	-0.10
<i>age (years)</i>						
0/0	(76)	-0.02	0.11	-0.05	0.00	0.07
≥0/1	(28)	0.05	0.14	0.09	0.16	0.29
Total	(104)	0.05	0.07	-0.03	0.03	0.05
Control †	(29)	0.13	-0.09	0.07	0.09	-0.20
<i>smoking (packyears)</i>						
0/0	(76)	0.16	-0.24*	-0.12	-0.27*	-0.14
≥0/1	(28)	-0.04	0.00	0.03	-0.27	-0.07
Total	(104)	0.09	-0.16	-0.07	-0.26**	-0.11
Control †	(29)	-0.15	-0.25	-0.46*	-0.20	-0.09

r = correlation coefficient significant at *p=0.05 or **p=0.01; † Not exposed to dust

Figure 8.2. Mean release of TNF- α from blood monocytes in 1987 (open bars) and 1992 (solid bars). Data were obtained from 12 and 29 (1987 vs 1992) control subjects not exposed to dust, from 39 and 76 control miners exposed to dust, and from 27 and 28 miners exposed to dust who had coal workers' pneumoconiosis. All values are means (SEM) and are expressed in ng/ml. The alphabetic symbols depict spontaneous release of TNF- α (A), or release of TNF- α in response to 3 ng/ml LPS (B), 1,000 ng/ml LPS (C), 5 mg/ml coal mine dust (D), or 0.5 mg/ml silica (E). Testing for significance was with the Mann-Whitney U test; the asterisks indicate significant differences between 1987 and 1992 data (* $p < 0.05$; ** $p < 0.01$). nd = not determined.

Despite these differences in absolute values, the 1992 cross sectional comparison of release of TNF between controls not exposed to dust, control miners and miners with CWP confirmed our previous observations (Fig. 8.2). Again, release of TNF in the controls not exposed to dust was significantly lower than in the control miners upon stimulation with high endotoxin (1000 ng/ml) and stimulation with silica. At present also release of TNF in the controls not exposed to dust was significantly lower than the control miners and miners with CWP upon stimulation with coal mine dust (Mann-Whitney U-test $p < 0.05$). Contrary to previous findings, however, none of the stimuli resulted in significant differences in release of TNF between miners with or without CWP; moreover the spontaneous release of TNF was significantly higher in the controls not exposed to dust *v* both miners' groups (Mann-Whitney, $p < 0.01$).

TNF and profusion score

Figure 8.3 shows the individual TNF data of the total follow up group ($n=104$) upon stimulation with coal mine dust as a function of profusion score. As previously [Borm et al., 1988], a large variation in response of TNF upon stimulation with coal mine dust was observed in the healthy miners group.

Figure 8.3. Release of TNF- α in 1992 in ng/ml of peripheral blood monocytes of individual miners stimulated with coal dust as a function of 1992 chest radiograph classification.

Profusion categories are 0 (0/0), I (0/1), II (1/0, 1/1, 1/2), III (2/1, 2/2, 2/3), IV (3/2, 3/3), V (3/+, progressive massive fibrosis).

In miners in the early stages of CWP, release of TNF was significantly increased and a decrease in release of TNF was found along with the increasing stage of CWP that seems to continue in PMF. Spearman rank correlation tests showed a significant downward trend in release of TNF with increasing profusion score starting from the ILO score 0/1, upon stimulation with coal dust ($n = 28$, $\rho = -0.41$, $p < 0.05$), silica ($n=28$, $\rho = -0.39$, $p < 0.05$) and to a lesser degree with low endotoxin (3

ng/ml, $n=28$, $\rho=-0.32$, $p<0.1$). In the HRCT subgroup ($n=46$) a downward trend of release of TNF was also found with increasing visual score of lesions in miners starting from ILO class 0/1; however this trend did not reach significance for any stimulus ($n=13$, $p>0.1$). In miners without the classic ILO evidence for CWP the release of TNF was not related to HRCT score, except for release of TNF stimulated with high endotoxin (1000 ng/ml). Interestingly, this increased with increasing number of lesions (Spearman, $\rho=0.35$, $p<0.05$, $n=33$).

Table 8.3 Group characteristics of the miners involved in the TNF study of 1987 ($n=66$) and the subgroup involved in the follow up ($n=40$).

	1987 ($n=66$)	1992 ($n=40$)
Age (years)*	49.4 (0.76)	48.1 (1.04)
Smoking (cigarettes/day)*	6.1 (1.00)	4.8 (1.15)
Smokers (yes/no)*	32/34	17/23
Medication (yes/no)*	10/56	6/34
Years underground (in 1987)*	21.2 (0.49)	20.7 (0.70)
Years underground (in 1992) [†]	22.3 (0.41)	21.9 (0.57)
Time retired (months) [†]	45.9 (2.00)	43.7 (2.75)
I.L.O. classifications [‡]		
0/0	39	25
0/1	7	4
1/0, 1/1, 1/2	9 (=2, 5, 2)	5 (=1, 2, 2)
2/1, 2/2, 2/3	8 (=0, 6, 2)	4 (=0, 4, 0)
3/2, 3/3	3 (=0, 3)	2 (=0, 2)
Release of TNF (ng/ml) [§]		
Spontaneous	0.86 (0.14)	0.93 (0.21)
Endotoxin (3 ng/ml)	3.72 (0.31)	3.65 (0.34)
Endotoxin (1000 ng/ml)	6.00 (0.59)	6.06 (0.77)
Coal mine dust	1.32 (0.20)	1.17 (0.16)
Silica	1.35 (0.20)	1.12 (0.19)

Values are mean (SEM); *data from the 1987 study; [†]data from personal records in the mine (1992);

[‡]chest radiograph in 1987; [§]see text for details

LONGITUDINAL DATA ANALYSIS: RESULTS OF FIVE YEAR FOLLOW

Follow up characteristics

Ultimately 104 coal miners (67% of the original cohort) participated in our follow up (Fig. 8.1) and 40 out of 66 coal miners involved in the TNF study [Borm et al., 1988] attended our follow up measurements. No selection bias was found for age, smoking, or time underground as was found in the original study (Table 8.3). Both groups did not differ for medication (yes/no, χ^2 -test), or for pneumoconiotic stage (1987 chest radiograph, χ^2 -test), or for mean release of TNF (Table 8.3). During the follow up interval all miners had left active mining and there had been no occupational exposure for at least four years in half of our miners and at least for two years in more than 85% of our miners. No selective loss to follow up from active mining had been taken place (Table 8.3). The mean (SD) age of retirement in our total follow up group (n=104) was 44.7 (5.1). Despite retirement a significant proportion of the cohort (n=104) showed progression of CWP. Based on the paired readings of the chest radiographs, it was shown that during this five year follow up period four out of 80 healthy coal miners contracted CWP (5%), and that in 11 cases existing CWP progressed (46%). In two miners also progressive massive fibrosis (PMF) was established (3/2rA and 2/3rA). From the miners of the original TNF study who were involved in the follow up study (n=40), one out of 30 control miners developed new CWP, and five out of 10 miners had progression of already existing CWP.

Longitudinal analysis of TNF in the follow up group

In the TNF follow up group, paired tests of the individual data of 1987 and 1992 (n=40), showed significant differences in spontaneous release of TNF, release of TNF upon stimulation with low endotoxin and with coal dust ($p < 0.05$). Significant correlations between the TNF data of 1987 and 1992 were found upon stimulation with coal mine dust ($r = 0.44$, $p < 0.01$) or low dose endotoxin ($r = 0.35$, $p < 0.05$). These correlations were also significant in the miners without CWP (n=28, $p < 0.01$, $r = 0.49$), and in all subjects (control miners and miners with CWP) without progression during the five year follow up (n=33, $p < 0.05$, $r = 0.43$) for stimulated release of TNF with coal dust as well as with 3 ng/ml endotoxin (data not shown). Upon stimulation with 1000 ng/ml endotoxin no significant correlation was observed in the whole TNF follow up group, but interestingly, a significant relation was found only in the six miners with five year progression of CWP (n=6, $p < 0.05$, $r = 0.89$). Correlations in the spontaneous release of TNF, or the release upon stimulation with silica were not found in any (sub)group.

TNF, exposure and progression

Multiple linear regression demonstrated that TNF is not a marker of exposure as release of TNF in either 1992 or 1987 was not related to individual cumulative (dust or quartz) exposure or to any of the other exposure variables in the TNF

follow up subgroup ($n=40$). Retrospective analysis showed, however, that the cumulative exposure was significantly related to progression of CWP. The odds ratio of cumulative exposure *v* progression of CWP was 3.72 (significant at a 90% C.I.), with the mean cumulative exposure (100 gh/m³) as a cut off for high and low exposure. In the TNF follow up subgroup ($n=40$), cumulative exposure was also significantly related to progression at various cut off levels. Figure 8.4 shows both these observations and that all but one miner who showed progression (or development) of pneumoconiosis between 1987 and 1992 originally had a release of TNF that exceeded the normal range in 1987. The relative risk of progression in CWP for miners who showed abnormal *in vitro* release of TNF was 8.1 (1.2-55.0, 90% C.I.), with the cut off for dust stimulated release of TNF of 2 SDs above the mean of the controls not exposed to dust.

Figure 8.4. Graphical illustration of the relative risk for miners with high ($> \text{mean} + 2 \times \text{SD}$) release of TNF- α (1987) and high ($> \text{mean}$) cumulative exposure to dust in the progression of CWP compared with all miners involved in the TNF- α follow up study ($n = 40$). Note that no correlation is present between TNF- α and exposure ($r^2=0.069$) and that the solid circles, which indicate the individuals with progression ($n=6$), are in the right quadrants (high TNF- α and high exposure) of the scheme. The arrow indicates the subject with first diagnosed CWP. In the upper right quadrant, age, smoking habits, cumulative exposure and 1987 or 1992 TNF- α release were not different between the miners with progression of CWP ($n=4$) and those without progression ($n=7$).

To determine sensitivity and specificity at any cut off point of TNF upon the different *in vitro* stimuli, ROC curves were calculated [van der Schouw et al., 1993]. Figure 8.5 shows ROC curves for spontaneous release of TNF and for release of TNF upon stimulation with 3 ng/ml endotoxin and coal dust. The strongest association between high TNF and progression was found release of TNF stimulated by dust, with an area under the curve (AUC) of 74%. No clear associations were observed in all other options (44%<A.U.C.<55%).

Figure 8.5. Receiver operator characteristic (ROC) curves of monocyte release of TNF- α in 1987 as indicator for the progression of CWP. The diagonal with an area under the curve (AUC) of 50%, marks the theoretical relation of an indicator without discriminative power. A perfect ROC association would be an inverse L-shaped curve in the upper left corner of the figure (AUC = 100%). The area under the curve was 44% for spontaneous release of TNF (sp), 55% for the release of TNF upon stimulation with 3 ng/ml endotoxin (end 3), and 74% for release of TNF upon stimulation with coal dust (Dust).

Discussion

The findings of our follow up study among retired coal workers lend support for an essential role of TNF in the genesis of pulmonary fibrosis induced by mineral dust in general [Elias et al., 1990; Perkins et al., 1993; Piguët et al., 1990; Driscoll et al., 1990; Mohr et al., 1991; Warren et al., 1988; Everson and Chandler 1992] and CWP in particular [Borm et al., 1988; Lasalle et al., 1990; Gosset et al., 1991; Vanhée et al., 1993]. Moreover this study shows that, when carefully applied, release of monocyte TNF is a valid tool for the prognosis of CWP. The large variation of release of TNF found in healthy miners (0/0) and miners at early stage of CWP (0/1) proved to be a scientific basis for a susceptibility marker: those with a high release of TNF upon stimulation with coal dust are at higher risk of development or progression of CWP. Although to test this hypothesis only analysis of the relation of the original release of TNF concentrations with prospective changes in CWP was needed, TNF was also measured in present study, and several other aspects were included. This also allowed us to test the possible feedback mechanisms in the progression of CWP, as already suggested, the reproducibility of the assay and cross sectional results, and effects of the end of occupational exposure (retirement). Individual exposure assessment and HRCT screening, both mainly focused on miners with normal chest radiographs, were included to investigate (or exclude) the role of *in vitro* stimulated release of TNF as a marker of exposure or as a marker of early (fibrotic) effects.

Cross sectional findings of the present study were similar to those of the original study [Borm et al., 1988]. Although significant differences in absolute TNF levels were found, release of TNF upon stimulation with coal dust and the low concentration of endotoxin in 1987 and 1992 were significantly correlated. This relation was present in the entire follow up group, as well as in all subjects without progression of CWP. Interestingly, TNF concentrations were not correlated in miners with progression of CWP, except for TNF released upon stimulation with the high concentration of endotoxin (n=6). This suggests feedback on release of TNF during active fibrosis. In contrast with our previous observations, no significant differences in TNF were found between miners with and miners without CWP, and the spontaneous release of TNF was significantly higher in the non-exposed controls. Both changes are likely due to the retirement of the miners in our present study—that is a reduced *in vivo* stimulation or priming. This is supported by findings of Lasalle et al [1990] in active and retired miners. These authors found that baseline release of TNF in alveolar macrophages was significantly higher in active miners. We therefore suggest that serum concentrations of TNF and baseline (spontaneous) release from monocytes or alveolar macrophages reflect an acute response to inhaled dusts, whereas the *in vitro* release by peripheral blood monocytes shows the chronic adaptation of a subject. In our study such an adaptation may be due both

to long term (and subsequent end of) exposure to coal dust and the effect of early pathogenic processes. Evidence for the involvement of exposure is the large variation of release of monocyte TNF in miners *v* controls. The role of early disease also seems evident, considering that the highest release of TNF was found in miners in the early stages of CWP.

Although release of TNF was highest in the early stages of CWP according to chest radiographs, HRCT analysis showed that release of TNF by men within this category was not related to the number of lesions found by HRCT. Therefore the variation in *in vitro* release of TNF, the basis of evaluation of this biomarker, is unlikely to be biased by early fibrotic processes. Also all evidence that could show that TNF is an exposure marker is negative; no relation between release of TNF and any exposure index (cumulative exposure, exposure years, time since first exposure) could be shown. This does not mean, however, that TNF is not affected by exposure, as TNF concentrations in coal miners are clearly different from those in subjects without chronic exposure to mineral dust. Perkins *et al.* reported similar findings in subjects with long term exposure to asbestos without evidence of asbestosis [Perkins *et al.*, 1993]. The lack of relations between release of TNF and exposure indices could be considered as a false negative result because of the original selection of subjects in the first case-control design [Borm *et al.*, 1988], matched on age and years underground, or to errors in the estimation of individual exposure. One could suggest that differences in exposure are therefore too small to detect any significant correlation with other variables. This is, however, contradicted by the large variation of cumulative exposure (four fold) at the same period of years underground, and the biological plausibility of the existing relations between exposure and CWP: calculated cumulative dust exposure correlated significantly with pneumoconiotic stage ($r=0.34$, $p<0.001$, chest radiographs; $r=0.44$, $p<0.005$, HRCT), and was significantly higher in miners with progression of CWP.

Interestingly, the incidence of CWP and progression to more severe stages in this small cohort was significant, especially when taking into account that all miners retired early during the follow up. As progression was derived from paired readings of chest radiographs, no doubt exists on the identity of progressed cases. The extent of progression is subject to variation due to differences in size of some radiographs, increased weight of most subjects, and interpretation error. Our findings are in line with epidemiologic data showing that progression is highest in advanced stages of CWP [Attfield and Wagner, 1992] and that incidence of CWP is more frequent among men who have left industry than among those in the active work force [Soutar *et al.*, 1986; Mahieu, 1990]. Therefore, there are extensive populations at risk of CWP throughout the world and methods of screening and surveillance by careful monitoring of the health of these miners and ex-miners are needed. In our study, both cumulative dust exposure and release of TNF in 1987 were significantly related to five year

changes in CWP. The (retrospective) estimate of the odds ratio for cumulative exposure was 3.72, and the relative risk for progression of CWP for someone with an abnormal ($> \text{mean} + 2 \text{ SD}$) release of TNF was 8.1 in this cohort. However, there is a subtle difference: dust exposure determines the prevalence of CWP after several years, whereas release of TNF determines the individual risk at a certain exposure (Fig. 8.4).

It should be emphasized that *in vitro* release of TNF induced by coal mine dust was the best predictor of progression, whereas baseline release of TNF was not related to five year progression of CWP (Fig. 8.4). Again this raises the question of priming of the monocytes by external exposure to mineral dust. Apart from our original suggestion of this phenomenon in coal miners [Borm et al., 1988], priming of macrophages was reported by Mohr *et al.* in rats exposed to silica [Mohr et al., 1991], coal miners [Lasalle et al., 1990] and asbestos workers [Perkins et al., 1993]. Local lung modulatory factors have been suggested in the upregulation of TNF secretion by monocytes [Pantelidis et al., 1993] but also autocrine pathways may be involved in the priming of macrophages [Tanner et al., 1992; Pfeffer et al., 1993]. We think that priming of monocytes in coal miners reflects the response to chronic release of factors from the lung loaded with coal dust. Studies with alveolar macrophages, and lung tissue of coal miners [Lasalle et al., 1990; Vanhe et al., 1993] confirmed this hypothesis and also suggested that as well as silica other compounds in coal dust and smoking [Lasalle et al., 1990; Gosset et al., 1991; Dubar et al., 1993] are important in the release of such factor(s). For example, in our study stimulated release of TNF was lower in miners who smoked, whereas the baseline response tended to be higher in non-smokers. Several results suggested the presence of feedback mechanisms that counteract the processes (dust exposure, smoking and inflammatory response) that upregulate monocyte release of TNF. Previously, and at follow up, we noted a downward trend of stimulated release of TNF as CWP stage increased. Moreover, in 1992 release of TNF was not such a good predictor of progression of CWP as it was in 1987. Also correlations between assays in 1987 and 1992 were only significant in miners without progression of CWP and not miner whose disease had progressed. We could not test the possible effect of feedback on TNF as a marker for progression of CWP in our cohort, because only retired miners were involved. Therefore, we are not able to discriminate between the effects of stopping exposure and failure of feedback as determinants of monocyte release of TNF. This remains an interesting subject for future investigations in a larger cohort of active miners.

In conclusion, our data add *in vivo* human evidence to the already existing *in vitro* and animal data that TNF is a crucial mediator in lung disease induced by mineral dust. It is appreciated that the size, original selection, and follow up interval of our cohort could limit the significance of our findings. The original

study had a case-control design matched for age and exposure to investigate the involvement of TNF in CWP. To reach acceptable response and statistical power (cases with progression), the cohort had to be supplemented with other miners, screened by us for different purposes in the same year. Nevertheless, the reproducibility of cross-sectional findings was excellent. Moreover, we showed that TNF data are not biased by either exposure or stage of disease and showed a high relative risk of high release of TNF at equal exposures and radiological outcomes. Its specificity as a marker is illustrated by the fact that none of the subjects with a normal release of TNF showed progression of disease. Determination of the ROCs (Fig. 8.5) showed that the significance of our findings was not solely due to the specific choice of the cut off in the present study, and allows the applicability of this marker in other cohorts of (retired) coal miners. Future follow up will increase the number of cases as the average age of our cohort is well below the current average to contract CWP [Soutar et al., 1986; Mahieu, 1990] and can help to get a more reliable estimate of the relative risk. Other estimates can come from studies that will use this method as a tool for health surveillance in, for example, miners who leave the active work force.

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Chapter 9

PLASMA LEVELS OF THE SOLUBLE TUMOUR NECROSIS FACTOR (TNF) RECEPTORS ARE INCREASED IN COAL MINERS.

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Abstract

Among other cytokines, Tumor Necrosis Factor- α (TNF) is considered to play a key role in the development of mineral dust related fibrosis. Previously, we showed that *ex-vivo* release of TNF by peripheral blood monocytes is a marker for progression of coal workers' pneumoconiosis (CWP). Since soluble TNF receptors (sTNF-Rs) are believed to play an important regulatory role in systemic effects of TNF, we measured plasma levels of sTNF-R55 and sTNF-R75 in coal miners with (n=28) or without (n=76) CWP and in non-exposed controls (n=29). sTNF-R75 levels were significantly increased in miners with CWP (2.09 ± 0.44 ng/ml) versus the non-exposed controls (1.86 ± 0.23 ng/ml). Neither sTNF-R55 nor sTNF-R75 were related to exposure, stage of pneumoconiosis, smoking, or (spontaneous or *ex-vivo* induced) monocyte TNF-release. sTNF-R55 was increased in subjects with medication (especially those using cardiovascular drugs); upon exclusion of these subjects, also sTNF-R55 was found also to be significantly increased in CWP. In conclusion, bearing in mind a confounding effect of medication, soluble TNF receptors are elevated in plasma of retired miners with CWP. These observations further support the important role of TNF-mediated pathways in the pathogenesis of mineral dust related fibrosis.

Introduction

Occupational exposure to mineral dusts can lead to pulmonary fibrosis [Crystal et al., 1991]. The pathogenesis of lung fibrosis involves highly complicated processes of intercellular communication by peptides released from and to various immune cells and lung target cells in a phenomenon appropriately referred to as "cytokine network" [Elias et al., 1990]. Previously, we and others showed that silica as well as coal mine dust, can stimulate the release of monocyte-/macrophage derived proinflammatory cytokines, such as Tumor Necrosis Factor- α (TNF) [Borm et al., 1988; Driscoll et al., 1990; Gosset et al., 1991], Interleukin-1 β (IL-1) [Driscoll et al., 1990; Schmidt et al., 1984; Lassale et al., 1990], Interleukin-6 (IL-6) [Gosset et al., 1991] and the Macrophage Inflammatory

Interleukin-6 (IL-6) [Gosset et al., 1991] and the Macrophage Inflammatory Proteins 1 α and 2 [Driscoll et al., 1992]. With regard to dust exposure in man we found that monocyte TNF-release upon *ex-vivo* stimulation with coal dust and silica (and endotoxin) was increased in (retired) miners with or without radiological evidence for simple coal workers pneumoconiosis (CWP) compared to subjects never exposed to mineral dust [Borm et al., 1988; Schins and Borm, 1995]. In line with our observations in monocytes of coal workers, higher TNF secretion was also observed in macrophages of patients with progressive massive fibrosis (PMF) compared to simple pneumoconiosis patients and control subjects [Lassale et al., 1990]. More recently, elevated mRNA levels of TNF (and IL-6) have been observed in lungs of pneumoconiosis patients [Vanhée et al., 1993]. Crucial evidence for the importance of TNF in silica-induced lung fibrosis was demonstrated by Piguet *et al.*, who showed a downregulatory effect on fibrosis by TNF-antibody treatment [Piguet et al., 1990].

Two distinct receptors of TNF, i.e. a 55 kDa type (TNF-R55) and a 75 kDa type (TNF-R75) have been identified, and both receptors are expressed on many different cell types and tissues [Brockhaus et al., 1990; Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990]. Shedding of the extracellular parts of these TNF receptors from white blood cells and probably also other cell types leads to two soluble TNF receptor types, known as sTNF-R55 and sTNF-R75 [Jäätelä et al., 1990; Beuteler et al., 1993]. Since naturally occurring soluble TNF receptor levels are believed to play an important regulatory role in systemic effects of TNF [Jäätelä et al., 1990; Sheperd, 1991] plasma TNF receptor levels may represent a feedback mechanism to the proinflammatory systemic action of TNF, or an (in)direct reflection of TNF-related pathological mechanisms within the lung. In the present study, we determined soluble TNF receptors in plasma of coal miners with or without CWP and in controls never occupationally exposed to dust. The purpose of the study was to evaluate the relation of soluble TNF receptors with cumulative (silica-) dust exposure and the biological effects of pneumoconiosis disease [Borm, 1994]. Secondly, it was designed to evaluate the relation between soluble TNF receptor levels and monocyte TNF release [Schins and Borm, 1995; Borm, 1994] in non-exposed subjects and in coal workers either in the presence or absence of lung fibrosis.

Materials and methods

As a part of a prospective cohort study among coal workers [Schins et al., 1994], soluble receptors level were determined in the plasma of coal miners (n=104) and non dust-exposed controls (n=29), after written informed consent. Prior to the blood sampling, a questionnaire, including informed consent was obtained from each participant. The study was conducted according to the Helsinki

declaration of 1975. On the day of blood sampling, a chest radiograph was made of each miner. Confirmation of questionnaires and data from medical files and job history were obtained by personal interviews. Subjects were classified as smokers, non-smokers, former smokers or life time nonsmokers; the amount smoked was expressed in pack years (packs/week x years smoked). Smoking habits were verified using a personal interview and smoking data from previous cross-sectional studies [Borm et al., 1990; Janssen et al., 1992] to minimize reporting bias. The same was done for medical history: All subjects were divided into two categories, i.e. those who used any kind of medication and those who did not use medication at least three days (or in the case of antibiotics: one week) prior to blood sampling.

Severity of pneumoconiosis was obtained by classification of chest radiographs by three occupational physicians [Schins et al., 1994], according to the standard protocol of the International Labour Organization [ILO, 1980]. High resolution computed tomography (HRCT) was performed in a subgroup of 46 miners on a voluntary basis as described previously [Lamers et al., 1994]. The cumulative dust exposure was determined from job-exposure matrices as described previously [Jorna et al., 1994]. Blood (10 ml EDTA-tube) was sampled, transferred to the lab at 4°C, and subsequently centrifuged. Plasma was stored at -70°C. Soluble receptor levels of R-55 and R-75 were determined by sandwich ELISA as described by Leeuwenberg [Leeuwenberg et al., 1994]. Briefly, immunoassay plates (Nunc Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with monoclonal anti-TNF-R55 or anti-TNF-R75. Plates were saturated with 1% BSA in PBS (w/v) for 1 h at room temperature. Test samples were added to the plates and incubated for 2 h at room temperature. Plates were washed and subsequently incubated with biotin-labeled rabbit anti-sTNF-R antiserum for 1h, followed by washing of the plates and addition of peroxidase labeled streptavidin. Peroxidase activity was determined spectrophotometrically after addition of 3,3',5,5'-tetramethylbenzidine substrate at 450nm by ELISA-reader. Present ELISA methods show no interference between sTNF-R and TNF, as previously reported [Leeuwenberg et al., 1994]. In the same subjects, monocyte TNF release was determined as reported previously [Schins and Borm, 1995; Borm, 1994].

Statistical analysis.

Study group characteristics were evaluated by the Student's t-test. Relations between the soluble TNF receptors R-55 and R-75 and age, pack years, and exposure (i.e. miners only) were evaluated by linear regression. Multiple comparisons were evaluated by stepwise regression analysis. All statistical evaluations were made using STATGRAPHICS version 6 (Manugistics Inc., Rockville Maryland 20852. USA).

Results

In the plasma of all individuals, levels of both soluble receptors were readily detectable (i.e. >200 pg/ml). Mean and standard deviations were 1.44 ± 0.44 ng/ml (sTNF-R55) and 1.97 ± 0.46 ng/ml (sTNF-R75). Plasma levels of sTNF-R75, but not sTNF-R55, were significantly elevated in miners with pneumoconiosis compared to subjects never exposed to dust (Table 9.1). Age in the non-exposed subjects was not different from miners with CWP, but was higher than in miners without radiological signs of pneumoconiosis (Table 9.1). Cumulative exposure was higher in miners with CWP compared to the reference miners. Plasma levels of sTNF-R55 and sTNF-R75 were not different between current, former or never smokers in any of the subgroups, and not related to the severity of pneumoconiosis as defined by conventional chest radiograph or by high resolution computed tomography (data not shown).

With the exception of age no linear correlations were present between the soluble TNF receptors and other variables as smoking, exposure, and monocyte TNF release (Table 9.2). A stepwise multiple regression analysis on both soluble TNF receptors using age, cumulative exposure (miners only), pack years smoked, medication, spontaneous and coal dust- stimulated monocyte TNF release as independent variables was performed in each study group. In the reference miners both age ($t=2.39$, $p<0.025$) and medication ($t=-2.32$, $p<0.025$) gave a significant fit in the model to sTNF-R55, but in the non-exposed controls only medication ($t=-3.12$, $p<0.005$) was significantly related to sTNF-R55. Subjects using medication had increased plasma levels of sTNF-R55 compared to subjects without medication (Table 9.3). Among the reference miners (but not in other groups), age was significantly higher in those having medication (Table 9.3), thus explaining the correlation between age and plasma sTNF-R55 levels in this subgroup. None of the variables was related to sTNF-R75 levels in any of the subgroups. Moreover, no relation was found between plasma levels of sTNF-R55 or sTNF-R75 and (spontaneous or induced) monocyte TNF release (Table 9.2).

Because of the significant effects of medication on sTNF-R55 levels, statistical analysis was repeated after exclusion of all subjects using medication. The difference in sTNF-R75 between CWP miners and non-exposed controls remained significant (Fig. 9.1, panel B), but now also an increase ($p<0.06$) of sTNF-R55 was observed in miners with CWP. This confounding effect of medication on sTNF-R55 and the increased tendency in sTNF-R55 in pneumoconiosis is illustrated in Fig. 9.1 (panel A). Again in this analysis no significant correlations were present between the soluble receptors and smoking or exposure, nor was a correlation observed with age. The previously significant correlation with age in the reference miners ($n=76$), was not present in the references without medication ($n=55$, $p>0.25$). Again, no relation between soluble

receptors and monocyte TNF release was seen in subjects not using medication (data not shown).

Table 9.1 Study group characteristics and plasma levels of the soluble TNF receptors sTNF-R55 and sTNF-R75 in miners with coal workers pneumoconiosis, reference miners and a non dust-exposed control group.

			Controls Nonexposed	Coal miners References	Coal miners with CWP
			(n=29)	(n=76)	(n=28)
Age	yrs		50.3 ± 5.2 *	47.7 ± 5.0	49.9 ± 5.6
			42 - 59	37 - 60	41 - 60
Smoking	pack-yrs		102.8 ± 100.5	110.8 ± 106.5	150.4 ± 128.5
			0 - 465	0 - 520	0 - 476
Medication	- no	n	21	56	21
	- yes†				
	(I)	n	0	5	2
	(II)	n	2	4	0
	(III)	n	1	3	1
	(IV)	n	0	4	2
	(V)	n	4	5	1
	(VI)	n	1	2	1
Cumulative dust exposure	ghm ⁻³	-		91.1 ± 56.4	130.9 ± 49.5 **
				10 - 280	23 - 203
sTNF-R55	ng/ml		1.47 ± 0.48	1.39 ± 0.43	1.52 ± 0.44
			0.78 - 2.55	0.36 - 2.67	0.96 - 3.08
sTNF-R75	ng/ml		1.86 ± 0.23	1.96 ± 0.53	2.09 ± 0.44#
			1.47 - 2.51	1.05 - 3.68	1.44 - 3.35

All values (i.e. mean ± standard deviation and *range*) are evaluated by the Student's t-test.

*p<0.05 and **p<0.01 significantly different from reference miners; #p<0.01 significantly different from non dust-exposed controls; CWP = miners with coal workers' pneumoconiosis (ILO chest radiograph >0/0); † Subjects were classified according to six treatment categories: (I) Inhalation drugs, i.e. β-agonists, corticosteroids, and theophylline (n=7); (II) cardiovascular drugs, i.e. b-blockers and diuretics (n=6); (III) Immunosuppressive and anti-inflammatory drugs and allopurinol (n=5); (IV) antibiotics (n=6); (V) H₂-agonists, laxantia, anti-psychotica and thyroid function drugs (n=10); (VI) others (n=4). In reference miners three subjects used two types of medication.

Table 9.2 Correlation of plasma levels of the soluble TNF receptors sTNF-R55 and sTNF-R75 with age, smoking, exposure, and monocyte TNF release.

	Soluble receptor	Controls Nonexposed (n=29)	Coal miners References (n=76)	Coal miners with CWP (n=28)
Age yrs	sTNF-R55	0.02 (0.92)	0.34 (0.03)	0.20 (0.30)
	sTNF-R75	0.12 (0.54)	0.19 (0.10)	-0.18 (0.37)
Smoking pack-yrs	sTNF-R55	0.19 (0.32)	0.08 (0.51)	-0.09 (0.64)
	sTNF-R75	0.10 (0.60)	0.00 (0.99)	-0.04 (0.83)
Underground working yrs	sTNF-R55	-	0.19 (0.11)	-0.16 (0.43)
	sTNF-R75	-	0.16 (0.18)	-0.06 (0.77)
Cumulative exposure ghm ⁻³	sTNF-R55	-	0.09 (0.45)	0.10 (0.60)
	sTNF-R75	-	0.04 (0.70)	0.06 (0.75)
TNF release ng/mL				
Spontaneous	sTNF-R55	-0.09 (0.66)	0.13 (0.26)	-0.06 (0.76)
	sTNF-R75	-0.05 (0.79)	0.11 (0.35)	-0.08 (0.70)
LPS, 3 ng/mL	sTNF-R55	0.18 (0.35)	0.17 (0.14)	-0.03 (0.90)
	sTNF-R75	-0.11 (0.59)	0.15 (0.19)	-0.11 (0.57)
LPS, 1000 ng/mL	sTNF-R55	0.04 (0.84)	0.06 (0.60)	-0.05 (0.80)
	sTNF-R75	-0.17 (0.39)	0.09 (0.42)	-0.02 (0.90)
Coal dust, 5 mg/mL	sTNF-R55	0.33 (0.08)	0.06 (0.63)	0.18 (0.36)
	sTNF-R75	0.13 (0.51)	0.07 (0.52)	0.01 (0.93)
Silica, 0.5 mg/mL	sTNF-R55	0.35 (.06)	0.12 (0.29)	0.26 (0.18)
	sTNF-R75	0.09 (.66)	0.00 (0.96)	0.11 (0.58)

All values are linear correlation coefficients (p-values); CWP = miners with coal workers pneumoconiosis (ILO chest radiograph >0/0); LPS = Lipopolysaccharide endotoxin.

Table 9.3 Effect of medication on plasma levels of soluble TNF receptors, and its relation with age, smoking and cumulative dust exposure

	Unit	non-exposed controls		reference miners		miners with CWP	
		medication	no medication	medication	no medication	medication	no medication
		(n = 8)	(n = 21)	(n = 20)	(n = 56)	(n = 7)	(n = 21)
age	(years)	49.9 ± 5.9	50.4 ± 5.4	50.1 ± 5.4 *	46.9 ± 4.7	48.7 ± 6.1	50.3 ± 5.5
smoking	(pack years)	139 ± 90	106 ± 111	99 ± 87	115 ± 143	178 ± 109	141 ± 136
cumulative exposure	(ghm ⁻³)	-	-	101 ± 67	88 ± 52	132 ± 43	131 ± 53
sTNF-R55	(ng/ml)	1.86 ± 0.65 **	1.32 ± 0.29	1.63 ± 0.50 **	1.30 ± 0.38	1.57 ± 0.35	1.50 ± 0.47
sTNF-R75	(ng/ml)	1.88 ± 0.18	1.85 ± 0.25	2.10 ± 0.54	1.91 ± 0.51	2.15 ± 0.62	2.07 ± 0.38

All values (i.e. mean and standard deviation) are evaluated by the Student's t-test; *p<0.05 and **p<0.01 significantly different from subject without medication in same subgroup; CWP = miners with coal workers' pneumoconiosis (ILO chest radiograph >0/0)

Figure 9.1. Effect of medication on plasma levels of the soluble Tumor Necrosis Factor Receptors (sTNF-R55 and sTNF-R75) in non-exposed controls (solid bars), reference miners (open bars), and miners with coal workers pneumoconiosis (dashed bars). Panel A represents receptor levels of subjects with medication (n=35); panel B shows all subjects without medication. Bars represent mean and standard deviations, evaluated by the Student's t-test. See table 9.1 for details on group sizes.

Discussion

To the best of our knowledge, this study is the first to describe a significant increase in plasma soluble TNF receptors in (simple) coal workers pneumoconiosis. sTNF-R75 levels were significantly increased in miners with CWP compared to the non-exposed controls. Controls (non-exposed), as well as reference miners, who used medication at the time of blood sampling also had increased levels of sTNF-R55, but upon statistical exclusion of these subjects, sTNF-R55 was also increased in CWP. No effect of smoking was seen and a relation of sTNF-R55 with age could be attributed to the effect of medication.

In the present study, no relation was found between monocyte TNF release and plasma levels of both soluble TNF receptors. Several reasons can explain for this lack of correlation. Firstly, it is unlikely that monocytes are the main source of sTNF-R in vivo. Secondly, monocyte TNF release was determined ex-vivo in standardized culture conditions, where cells were seeded at equal concentrations in culture dishes, while plasma sTNF-R levels might be related to individual variations in white blood cell concentrations. Unfortunately, no differential or total cell counts of the blood were available.

Since all miners in our study were retired, acute effects of coal dust exposure can be excluded. Moreover, plasma receptor levels were not related to cumulative dust exposure. In the miners with CWP (n=28), a clear relation with disease severity, as determined by conventional chest radiograph or HRCT could not be established (data not shown). From these observations, one might suggest that levels of sTNF-R55 and sTNF-R75 could play a role in the pathogenesis of CWP.

Although many cytokines and growth factors are known to be involved in pulmonary fibrosis, the role of the "early" proinflammatory cytokine TNF- α as a central mediator in mineral dust related fibrosis is underscored by several *in vitro* and *in vivo* data [Borm et al., 1988; Driscoll et al., 1990; Gosset et al., 1991; Lassale et al., 1990; Vanheé et al., 1993; Mohr et al., 1991; Perkins et al., 1993]. The recent discovery of soluble cytokine antagonists -and particularly the soluble TNF receptors R55 and R75- have put things in a new perspective: soluble cytokine receptors could, on the one hand, act as powerful antagonists of various cytokine actions, but, on the other hand, increase cytokine related effects by a systemic carrier function of shortlived cytokines [Beutler et al., 1993; Ulich et al., 1993]. Piguet *et al.* demonstrated a downregulatory effect of TNF-antibodies [Piguet et al., 1990], and more recently of sTNF-R55 [Piguet et al., 1994] in mineral dust related fibrosis. Doing so, they not only proved the crucial role of TNF in this disease, but they also elucidated a possible (therapeutic) role of soluble TNF receptors in mineral dust related fibrosis. Increased levels of soluble TNF receptors have been observed in several chronic and acute diseases [Aderka et al., 1991; Naylor et al., 1993; Latini et al., 1994; Shapiro et al., 1993; Tilg et al., 1993]. In chronic lung disease sTNF-R55 and R75 were found to be elevated in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis and sarcoidosis [Walker et al., 1994]. In line with our observations on monocyte TNF release in coal miners [Borm et al., 1988; Schins and Borm, 1995; Borm, 1994], sTNF-R55 and sTNF-R75 therefore also may play a role in the pathogenesis of coal workers' pneumoconiosis.

Unlike sTNF-R55, plasma levels of sTNF-R75 in the present study were clearly elevated in the plasma of miners with simple coal workers pneumoconiosis. Moreover, sTNF-R75 levels were not affected by medication. This might be in line with recent observations that TNF induced receptor shedding from peripheral blood neutrophils is specific to TNF-R75 [Porteu et al., 1994]. Similarly, the (slower) release of TNF receptors from mononuclear cells in response to LPS is a process also predominantly observed for TNF-R75 compared to TNF-R55 [Leeuwenberg et al., 1994].

From above data one might consider the applicability of plasma determination of soluble TNF receptors with regard to monitoring, as previously suggested for high *ex-vivo* monocyte TNF release as a positive risk factor for disease progression of CWP [Schins and Borm, 1995]. However, one should be aware of confounding factors on soluble TNF receptor measurements. Since the

kidney is believed to be the main clearance route of soluble TNF receptors, any abnormality in renal function would result in altered plasma levels of both receptors [Bemelmans et al., 1993]. Shapiro et al. have also stressed the importance of appropriate blood processing for sTNF-R determinations [Shapiro et al., 1993]. In the present study, we observed an effect of medication on plasma levels of sTNF-R55. Due to a limited number of subjects using medication, we can only speculate on the action of specific types of medication although higher plasma levels of sTNF-R55 were found in those subjects using cardiovascular drugs compared to subjects without medication ($p < 0.05$, $n = 6$). Whether (in)direct action of the drugs, or on the other hand the (history of) cardiovascular pathogenesis in these medicated subjects alters plasma sTNF-R55 kinetics (e.g. transcription, internalization, shedding, renal clearance) remains to be elucidated. However, it should be realized that in this small subgroup other effects may as well account for increased sTNF-R55 levels.

In conclusion, both sTNF-R55 and sTNF-R75 are increased in coal workers pneumoconiosis, although differences in receptor levels with exposed or non-exposed control groups may be confounded by the effect of medication used at time of blood sampling. The increased levels of soluble TNF receptors further support the important role of TNF in pneumoconiosis in coal workers. Interactions between TNF release and the antagonistic actions of soluble TNF receptors could be important in the development or progression of CWP. If so, measurement of these receptors may be of additional value in routine screening of dust exposed individuals. Support for this should be derived from a prospective study among coal workers, allowing a concomitant evaluation of TNF and its soluble receptors as risk markers.

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Chapter 10

MULTIPLE CYTOKINES AS BIOMARKERS IN COAL DUST EXPOSURE AND PNEUMOCONIOSIS: TNF- α , IL-6, AND TGF- β .

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Abstract

Previously we showed that the individual risk for progression of Coal Workers Pneumoconiosis (CWP) is related to abnormal release of Tumor Necrosis Factor- α (TNF) from monocytes stimulated *in vitro* with coal dust. In this study we investigated Transforming Growth Factor- β 1 (TGF) and Interleukin-6 (IL-6) in the same cohort, since in line with TNF (1) they are released from coal dust stimulated alveolar macrophages, and (2) their significance is demonstrated in animal models of (silica) fibrosis as well as in lung of CWP patients. Spontaneous as well as coal- and silica dust-stimulated release of TGF and IL-6 was evaluated by ELISA in the same monocyte supernatants (18 hours) previously used to determine TNF release, including the supernatants from non-exposed individuals (n=29), miners without CWP (n=76) and with CWP (n=28). As previously reported, coal- and silica-stimulated, but not spontaneously released TNF levels were higher in miners compared to non-exposed subjects. Spontaneous as well as coal dust- (5 mg/mL) and silica- (0.5 mg/mL) stimulated TGF release was significantly higher in miners compared to non-exposed individuals. However, TGF release from dust- or silica- stimulated monocytes was significantly lower than baseline release. IL-6 release was not different between the groups, and, unlike silica, stimulation with coal dust increased IL-6 release compared to spontaneous IL-6 release. Remarkably, dust- stimulated TGF and TNF release were positively correlated in the non-exposed individuals ($p=0.55$, $p<0.01$), but negatively correlated in the miners with CWP ($p=-0.55$, $p<0.01$). Multiple logistic regression analysis showed that specific patterns of these cytokines released from monocytes are related to chronic coal dust exposure as well as resultant pneumoconiosis. In particular, the relative release of TGF and TNF from coal dust stimulated monocytes seems a powerful marker of progression of CWP. Follow-up will elucidate the validity of these cytokines as multiple risk markers in CWP.

Introduction

Pneumoconiosis caused by chronic inhalation of mineral dusts including asbestos, silica and coal dust are among the most widespread occupational diseases [Cullen et al., 1990]. Chronic inhalation of coal dust may lead to coal workers pneumoconiosis (CWP), characterized by a relatively mildly interstitial fibrosis at early stage disease [Gross, 1983; Attfield and Wagner, 1992]. Against the background of this simple CWP, occasionally progressive massive fibrosis (PMF) may develop, characterized by large lesions, severe fibrosis and collapse of lung parenchyma [Flint, 1988]. Regarding their potent stimulatory or inhibitory actions, several cytokines have been considered to amplify, respectively counteract the development or progression of fibrosis in man [Elias et al., 1990]. In mineral dust induced fibrosis, the alveolar macrophage is considered to play an important role [Heppleston and Styles, 1967; Takemura et al., 1989], and in vitro stimulation of these cells with mineral dust particles results in the release of many factors, including Tumor Necrosis Factor- α (TNF), Interleukin-1 (IL-1), Interleukin-6 (IL-6), Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor- β (TGF), Insuline-like Growth Factor-I (IGF-I) [Schmidt et al., 1984; DuBois et al., 1989; Lasalle et al., 1990; Perkins et al., 1993; Vanhe et al., 1994].

In vivo and in situ studies in animal models and fibrotic patients have identified some crucial cytokines with respect to mineral dust induced fibrosis. The role of TNF was demonstrated by Piguet and colleagues, in a mouse-model of fibrosis, using bleomycin as well as - more specifically with regard to pneumoconiosis- using silica [Piguet et al., 1989; 1990]. In both models, addition of TNF antibodies resulted in a significant attenuation of fibrogenesis, while concomittant addition of recombinant TNF further increased fibrogenic response. TNF as well as IL-6 was found in most of the cells from the nodular lesions [Piguet et al., 1990]. More recently, mRNA levels of TNF and Interleukin-6 (IL-6) were found to be elevated in lungs of CWP and PMF patients [Vanhe et al., 1995]. Transforming Growth Factor- β (TGF) has also be found in bleomycin models of fibrosis [Raghow et al., 1989; Piguet et al., 1989], however unlike TNF, and IL-6, no elevated TGF was seen in the silica model [Piguet et al., 1990]. Furthermore, alveolar macrophages (obtained by bronchoalveolar lavage) from CWP patients were found to release increased amounts of TNF, IL-6, PDGF, TGF, and IGF-1 in vitro, compared to alveolar macrophages from control subjects [Gosset et al., 1991; Vanhe et al., 1995]. In line with these observations, alveolar macrophages of asbestos exposed subjects were found to produce increased levels of TNF, IL-1 and IL-6 [Perkins et al., 1993], and alveolar macrophages from rats exposed to silica release increased amounts of TNF and IL-1 [Driscoll et al., 1990].

Previously we evaluated spontaneous and stimulated TNF release from peripheral blood monocytes of coal miners in relation to chronic coal dust exposure and CWP [Borm et al., 1988]. In a longitudinal study we were able to

show that the risk for progression of CWP is related to individual TNF release [Borm, 1994; Schins and Borm, 1995]. In our cohort, miners with an abnormal high TNF release from monocytes stimulated with coal dust had a six-fold risk for disease progression over a five-year follow-up period [Schins and Borm, 1995].

Since peripheral blood monocytes are also important sources of TGF [Grotendorst et al., 1989] and IL-6 [Kotloff et al., 1990], we evaluated in the present study spontaneous as well as coal- and silica dust stimulated release of TGF and IL-6, in the same monocyte supernatants used to determine TNF release as previously reported [Schins and Borm, 1995]. Aims of the study were (a) to evaluate monocyte release of TGF and IL-6 in response to silica or coal dust, and (b) to study the inter-individual differences in cytokine release among non-exposed individuals versus subjects chronically exposed to coal dust, as well as in simple CWP. Overall patterns of cytokine release were analysed to evaluate their possible involvement in mineral dust exposure, and to determine which cytokines can be used as biomarkers of CWP, either independently, or in combination with TNF i.e. as "multiple markers" in coal dust induced fibrosis.

Methods

The study group consisted of 29 non-exposed individuals, 76 control miners and 28 miners with CWP. At the time of blood sampling, all coal workers had been retired [Schins et al. 1994]. An extensive questionnaire was sent to all participants in which medical complaints, medical history smoking habits, and medication were asked for. Presence and stage of CWP was evaluated by chest radiograph, scored according to I.L.O. classification rules [ILO, 1980] by an experienced panel of three physicians [Schins et al., 1994; Schins and Borm, 1995]. Miners were grouped as either "healthy coal workers" (HC, i.e. chest radiograph = 0/0) or as miners with evidence for Coal Workers Pneumoconiosis (CWP, i.e. chest radiograph $\geq$ 0/1). The control group consisted of 29, age matched, non dust-exposed, male subjects. Exclusion criteria for non dust-exposed control subjects were: (I) working for more than one year in occupational settings related to chronic dust exposure (e.g. mining, cement works, ceramic industry, bakery, etc.) and (II) one or more of asthma, (chronic) bronchitis and other chronic diseases of lung, liver and kidney. Exposure parameters were used were (I) time worked underground, (II) time since first occupational exposure, (II) time since last occupational exposure, (IV) cumulative exposure to respirable coal dust and quartz content [Schins and Borm, 1995].

Monocyte incubations and cytokine assays

Spontaneous as well as coal- and silica dust-stimulated release of TGF and IL-6 was evaluated in the same monocyte supernatants used to determine TNF release as previously reported [Schins and Borm, 1995]. Monocytes were isolated and stimulated as described previously [Schins and Borm, 1995]. Briefly, blood (3 x 10 ml, EDTA-tubes) was sampled and left overnight at room temperature. Mononuclear cells were isolated by buoyant density centrifugation (Lymphoprep-Nycomed®), adjusted to concentrations of 0.5×10^7 cells/ml and seeded (0.5 ml) to tissue culture dishes (Costar TC-24) to allow adherence (repetitive incubation, agitation and washing). Adherent monocytes were stimulated with coal dust (5 mg/ml) or silica particles (0.5 mg/ml). Each individual's baseline cytokine release was measured without addition of dusts. Monocytes were incubated in RPMI culture medium containing 5% FCS (Gibco, Life Technologies, Paisley, UK). After 18 hours of incubation (37°C, CO₂) the cell free supernatants were stored at -80°C until cytokine analysis. TNF was measured by TNF-specific sandwich-ELISA as described previously [Schins and Borm, 1995]. The detection limit of the assay was 10 pg/ml. Interleukin-6 was measured by ELISA as described by Dentener [Dentener et al., 1993]. The ELISA for IL-6 has a detection limit of 10 pg/ml. TGFβ1 (active as well as latent forms) was measured by ELISA as described previously [Vanhée et al., 1994].

Statistical evaluations

Correlations among cytokine levels and between cytokine levels and (cumulative) exposure, age, and smoking (pack years) were evaluated by Spearman Rank correlation. Differences between subgroups were evaluated by Mann-Whitney U-test. Multiple logistic regression (MLR) was used to evaluate the significance of either spontaneous or particle-stimulated monocyte release of TNF, TGF, and IL-6 (a) in relation to exposure (i.e. non-exposed versus healthy miners), (b) in relation to pneumoconiosis (i.e. healthy miners versus miners with CWP), and (c) in relation to (retrospective) 5-year progression of CWP. Models were evaluated upon correction for age (model a) or cumulative exposure (models b and c). All statistical evaluations were made using SPSS 6.1 (SPSS Inc. Chicago, IL).

Results

Study group characteristics are shown in Table 10.1. Cumulative exposure was significantly higher in miners with CWP, and age was significantly higher in the non-exposed control group, compared to miners without CWP (see Table 10.1). As previously reported, coal and silica dust stimulated (but not spontaneously released) TNF levels (18 hours) were higher in miners compared to non-exposed

subjects (see Figure 10.1 A). Silica stimulated TNF release was lower than coal dust stimulated release at the concentrations used. IL-6 release was not different between the groups, and unlike coal dust, stimulation with silica did not cause increased IL-6 release compared to spontaneous IL-6 release (see Figure 10.1 B). Remarkably in the control coal workers, spontaneous IL-6 release significantly exceeded silica stimulated release (Mann-Whitney, $p < .01$). In both miner groups, but not in the non-exposed individuals, coal dust induced IL-6 was significantly higher than IL-6 released in the presence of silica (Mann-Whitney, $p < .01$).

Table 10.1 Characteristics of the study cohort in 1992 (n=133).

	Non-exposed references (n=29)	Control miners controls (n=76)	Miners with CWP CWP (n=28)
Age (years)	50.3 (1.0) *	47.7 (0.6)	49.9 (1.1)
Smoking (packyears)	115 (20)	111 (12)	150 (24)
Cumulative dust (gh/m ³)	-	91.1 (6.4)	130.9 (9.3) **
Cumulative quartz (mgh/m ³)	-	4798 (306)	6751 (461) **
Years underground	-	22.2 (0.4)	25.0 (0.9) **
Years since first exposure	-	28.9 (0.6)	30.7 (1.0)
Months retired	-	46.3 (2.1)	53.9 (2.1)

Values are mean and s.e.m.; the asterisks denote a significant difference towards the control miners group: * $p < 0.05$ ** $p < 0.01$ (Mann-Whitney)

Spontaneous as well as coal and silica dust stimulated TGF release was significantly higher in miners compared to non-exposed individuals (see Figures 10.2 A and 10.2 B). However at the concentrations used, TGF release from dust or silica stimulated monocytes were significantly lower than their baseline release, except in the non-exposed control group. In this group only coal dust stimulated release was significantly lower. Furthermore, and significantly only in the non-exposed group ($p < .01$, Mann-Whitney), TGF tended to be lower in the coal dust incubations, than in the silica incubations. None of the cytokines was significantly correlated to exposure parameters or smoking, except active TGF in the silica stimulated supernatants, which was slightly higher in nonsmokers compared to smokers ($p < 0.05$, Mann-Whitney). Silica stimulated TGF release tended to decrease with increasing radiological profusion (Spearman $\rho = -0.39$, $p < 0.05$). Spontaneous TNF and (total) TGF release were inversely correlated ($\rho = -0.26$, $p < 0.01$), while TNF and IL-6 were positively related ($\rho = 0.41$, $p < 0.001$).

Figure 10.1. Spontaneous, coal dust stimulated, and silica stimulated release of TNF (panel A), and IL-6 (panel B) from monocytes of non-exposed references (n=29, open bars), control miners (n=76, hedged bars), and miners with CWP (n=28, solid bars). All values are evaluated by Mann-Whitney U-test. The asterisks indicate a significant difference versus the reference group at * $p<0.05$ and ** $p<0.01$ respectively.

Figure 10.2. Spontaneous, coal dust stimulated, and silica stimulated release of TGF from monocytes of non-exposed references (n=29, open bars), control miners (n=76, hedged bars), and miners with CWP (n=28, solid bars). All values are evaluated by Mann-Whitney U-test. The asterisks indicate a significant difference versus the reference group at * $p<0.05$ and ** $p<0.01$ respectively. Panel C represents TGF levels without acidification of the supernatants; panel D represents TGF levels upon acidification.

Correlations between TNF and IL-6 also reached significance in coal workers without CWP. Coal dust stimulated IL-6 release was positively correlated to TNF ($\rho=0.42$, $p<0.001$) and to TGF ($\rho=0.37$, $p<0.001$), and this relation was also significant in the non-exposed individuals. Remarkably, in the non-exposed individuals coal dust stimulated TNF and TGF were positively correlated ($\rho=0.55$, $p<0.01$) while in the miners with CWP a significant negative correlation was observed ($\rho=-0.55$, $p<0.01$). In control miners, correlation between TGF and TNF was absent.

Multiple logistic regression analyses (Table 10.2), showed that the release of TNF, IL-6 and TGF was significantly related to exposed (i.e. miners) versus non-exposed individuals as well as to pneumoconiotic versus healthy miners. Since age was higher in the non-exposed subjects (see Table 10.1), analysis was also performed upon correction for this parameter. Similarly, models were evaluated upon correction for cumulative exposure, as it was significantly higher in those with CWP (see Table 10.1), as well as those who had progression of CWP as reported previously [Schins and Borm, 1995].

Table 10.2 Multiple logistic regression analysis of spontaneous and dust stimulated monocyte release of TGF, TNF and IL-6 in relation to chronic coal dust exposure, and (progression) of CWP.

	Odds	[95 % C.I.]	Odds adjusted\$	[95 % C.I.]
Exposure (n=76 vs. n=29)				
spontaneous	18.89 *	[4.33 - 82.32]	27.08 *	[6.65 - 110.3]
coal dust	7.00 *	[1.99 - 24.66]	22.00 *	[5.33 - 90.57]
silica	2.69	[0.35 - 20.72]	2.50	[0.65 - 9.57]
CWP (n=28 vs. n=76)				
spontaneous	9.35 #	[0.91 - 95.96]	4.17 #	[0.99 - 17.51]
coal dust	-	†	5.36 *	[1.13 - 25.29]
silica	-	†	5.13 *	[1.16 - 22.64]
Progression (n=17 vs. n=87)				
spontaneous	-	†	-	†
coal dust	-	†	-	†
silica	-	†	4.36	[0.25 - 75.30]

\$ Odds ratio corrected for age (Exposure) or cumulative dust exposure (CWP, Progression), using multiple logistic regression; * sign at 95% C.I. ($p<0.05$); # sign at 90% C.I. ($p<0.10$); † Odds ratio not calculated; empty cells

These models were applied for spontaneous and stimulated cytokine release, and led to the Odds ratio's (95% confidence intervals) of the observed and predicted classification according to the models shown in Table 10.2. Spontaneous as well as coal dust stimulated patterns of cytokines release from monocytes were affected in reference miners compared to the non-exposed controls (i.e. related to chronic occupational exposure history). Furthermore, both spontaneous and dust stimulated release were related to the presence or absence of pneumoconiosis. Opposite to spontaneous release however, stimulated release was significant only upon adjustment for cumulative exposure.

Discussion

In the present study, we evaluated whether individual differences in (patterns of) cytokines released from peripheral blood monocytes, including TNF, TGF and IL-6 can be used as biomarkers in CWP. Independent analysis of the cytokines showed that TNF, TGF as well as IL-6 were affected by chronic dust exposure or pneumoconiotic disease (see Figures 10.1 and 10.2). As reported previously, coal- and silica dust stimulated TNF release was higher in miners compared to non-exposed subjects [Schins and Borm, 1995]. The new data show that both spontaneous and dust stimulated release of TGF are increased in monocytes of subjects chronically exposed to coal dust. However at the concentrations used, spontaneous release of TGF from monocytes was higher than the silica and the coal dust- stimulated release. These differences may be caused by inhibitory effects of other cytokines released during the incubations in response to these dusts, or by absorption of the TGF by dust. Support for the latter explanation was obtained from preliminary experiments using incubation of recombinant human TGF in the presence of coal dust (data not shown). The release of IL-6 from unstimulated monocytes, as well as from monocytes stimulated with coal dust, was generally correlated to TNF release. Unlike TNF however, dust stimulated IL-6 release was not increased, and spontaneous release was not decreased in (control) miners compared to non-exposed individuals. Furthermore, IL-6 release from monocytes incubated in the presence of silica did not exceed its baseline release. Bearing in mind that the silica content of the batch of coal dust (i.e. 6.4% /number or 6.2%/weight) at its concentration used (i.e. 5 mg/ml) did not exceed the concentration of silica used (i.e. 0.5 mg/ml), these differences might be related to differences in the hazard of silica versus coal dust [Gosset et al., 1991].

Although differences between the non-exposed references and the (control) miners were observed, none of the cytokines was related to cumulative dust exposure in the miners. These data are not in favour of the use of monocyte release of TNF, TGF or IL-6 as exposure markers in coal miners. Furthermore, no

relation was seen with radiological stage of CWP (conventional chest radiograph, HRCT), with the exceptions of silica stimulated TGF release and, as previously reported [Schins and Borm, 1994], coal dust stimulated TNF release, which both tended to decrease with increasing radiological stage of CWP. Finally, none of these cytokines measured was related to progression of CWP. However, it should be emphasized that present analysis was performed retrospectively, and that only prospective analysis by means of future follow-up of the present cohort will eventually elucidate the predictive value of present multiple cytokine models. Previously we have validated the release of TNF in such a prospective assay [Schins and Borm, 1995].

Based on these previous results and the present cross-sectional data we now consider combined measurement of multiple cytokines as powerful candidate markers in CWP. Indeed, multiple logistic regression analysis (see Table 10.2) clearly shows the benefits of the multiple marker interpretation of monocyte release of TGF, TNF and IL-6, in comparison to a "single marker" approach. However, the phenomenon of combined cytokine release from the same monocytes, also rises specific questions on the biological interpretation of these data. Cytokines released from the monocytes may be involved in autocrine amplification loops or in feedback mechanisms, as well as in synergisms or antagonisms with other cytokines or (soluble) receptor (ant)agonists released from the same monocytes.

The complexity of these cytokine network interactions [Elias et al., 1990] in the monocyte incubations is underscored by several observations in our study. First, in the spontaneous incubations, TNF and TGF were negatively correlated, while TNF and IL-6 were positively correlated. A correlation between spontaneous release of TNF and IL-6 supernatants from mononuclear cells from sarcoidosis patients, but not in the controls, has been recently reported [Prior et al., 1996]. In contrast, the correlation between TNF and IL-6 reached significance in our control miners only, but neither in the non-exposed references nor in the miners with CWP. Secondly, when considering dust stimulated monocytes, TGF and TNF were positively correlated in the non-exposed individuals, inversely correlated in subjects with CWP, while in control miners correlation was absent. In line with this observation one could suggest the presence of specific inter-individual differences in patterns of the relative release of TGF and TNF in relation to chronic coal dust exposure and/or resultant disease. To further evaluate this concept, individuals were classified into one out of four groups based on upper and lower tertiles of TNF and TGF levels (Table 10.3), excluding subjects with TNF and/or TGF release in the middle tertile. Age, cumulative dust exposure and smoking were not significantly different among these four groups (data not shown). Remarkably, none of the non-exposed individuals had high release of TGF, while only 3 out of 12 had high release of TNF, and none of

the miners with CWP showed either low release or high release of both TNF and TGF at the same time. A third observation emphasizing the complexity of the cytokine networks, is the difference in spontaneous versus stimulated release of the cytokines, considering that all miners involved in this study had been retired for considerable time [Schins and Borm, 1995]. Coal dust stimulated release of TGF and TNF were both elevated in the (retired) miners in comparison to the non-exposed individuals (see Figures 10.1 and 10.2). However, spontaneous release of TGF was approximately twice as high in the retired workers compared to non-exposed individuals, while spontaneous release of TNF was significantly lower in those formerly exposed (see Figures).

Table 10.3 Classification of non-exposed controls, reference miners, and miners with CWP versus individual release of TNF and TGF from dust stimulated monocytes

TNF/TGF		low/low	high/low	low/high	high/high
Non-exposed references	(n=15)	12	3	-	-
Control miners	(n=24)	2	6	9	7
Miners with CWP	(n=8)	-	3	5	-

\$ Subjects were grouped according to upper (= "high") and lower (= "low") tertiles of cytokine release: Group 1 = subjects having low TNF and low TGF release, group 2 = subjects having high TNF and low TGF release, group 3 = subjects having low TNF and high TGF release, and group 4 = subjects having high TNF and high TGF release.

One might speculate on the meaning or role of kinetic mechanisms, or different subpopulations of peripheral blood monocytes, between retired miners versus non-exposed individuals. Although peripheral blood monocytes are a major source for newly recruited alveolar macrophages (AM), it is not known as to how far these in vitro observations play a crucial role in CWP. Remarkably however, alveolar macrophages from PMF patients spontaneously release increased levels of TNF, but lower TGF as compared to subjects with (simple) CWP [Lasalle et al., 1990; Vanhe et al., 1995]. Macrophage supernatants of those with simple CWP, but not of those with PMF, and not if incubated in the presence of anti-TGF, inhibit fibroblast proliferation in vitro [Vanhe et al., 1995]. Furthermore, time course studies in (bleomycin induced) fibrosis demonstrated a shift in TGF source from AM at early stages of fibrosis to extracellular matrix in later stages [Khalil et al., 1989], as well as changes in TNF secretion related to changes in AM subpopulations [Everson and Chandler, 1992]. In line with these observations,

interestingly also subsets of peripheral blood monocytes are known to have considerable differences in cytokine releasing capacities [Frankenberger et al., 1996].

However, it should be realized that the observed patterns of cytokine release in the present study are also dependent on the concentrations of dusts used, as well as on the time of the incubations. In the present study, these experimental conditions (see methods section) are based on the original kinetic experiments to determine the optimal conditions regarding the release of TNF solely [Borm et al., 1988; Schins and Borm, 1995]. As such, the final concentrations of the cytokines measured in the supernatants of the unstimulated or dust stimulated monocytes, are the net result of the experimental conditions used (incubation time, concentrations of dust), but necessary also reflect inter-individual differences in kinetic response of each individuals monocytes. Irrespectively of the experimental conditions, two major observations can be made: (1) Monocytes of retired coal miners seem to be primed to release increased levels of TGF without any ex vivo challenge, while they need a specific stimulus (e.g. coal dust) to release increased TNF. (2) In vitro stimulation of monocytes of individuals (formerly) exposed to coal dust leads to an enhanced release of both TNF and TGF compared to monocytes of non-exposed references, while in those with pneumoconiotic disease, the enhanced release of either TGF or TNF disappears.

Finally, the spontaneous TGF release measured in the present study was found to be negatively correlated to glutathione concentrations in erythrocytes, isolated from the same individuals at the same day ($r = -0.25$, $p = 0.01$, $n = 117$). Moreover, we found in a prospective survey that reduced erythrocyte GSH levels measured at earlier time were a risk factor for the development of CWP [Schins et al., submitted for publication]. Since a downregulatory effect on GSH accumulation by TGF in vitro was recently reported [Arsalane et al., 1995], our findings provides indirect support for the involvement of monocyte TGF release and related red cell GSH in CWP.

In conclusion, our data show that in addition to TNF, also IL-6 and TGF play a role in coal dust exposure and/or resultant pneumoconiotic disease. Future follow-up of our study cohort will elucidate the validity of the combined evaluation of these cytokines in (ex) coal miners as susceptibility markers of CWP.

PART 3

Collagen

Chapter 11

SERUM PROCOLLAGEN TYPE III PEPTIDE IN COAL WORKERS' PNEUMOCONIOSIS: A FIVE YEAR FOLLOW-UP STUDY.

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Abstract

Several studies have suggested the use of serum type III procollagen peptide (PIIP) as a marker in fibrotic lung disease. To test serum PIIP as a biomarker for progression of coal workers' pneumoconiosis (CWP), a follow-up study was conducted among 156 coal miners. A five-year progression of simple CWP was determined from paired chest X-ray readings and related to serum PIIP measured in 1987 (n=73) and in 1992 (n=104). Although in eight subjects CWP had progressed, none of these subjects had abnormal levels of serum PIIP, nor were they different from non-progressed miners with regard to exposure, age or smoking habits. The results suggest that the use of serum PIIP as a biomarker to screen for coal workers' pneumoconiosis is limited.

Introduction

A crucial biological mechanism in pulmonary fibrosis is the (chronic) activation of fibroblasts and the subsequent excessive formation and deposition of collagen fibrils [Snyder, 1988]. During early stages of pulmonary fibrosis the ratio of collagen type III to collagen type I increases at active fibrotic sites [Bateman et al., 1981]. In the search for biological markers to better predict or estimate development, outcome, or activity of disease, determination of the serum levels of the procollagen type III peptide (PIIP) -a degradation product of the type III procollagen precursor- has been widely applied in the context of many diseases of the lung.

Several studies have established relations between PIIP levels in serum or bronchoalveolar lavage (BAL) and several lung diseases [Low et al., 1992; Cavalleri et al., 1991; Pohl et al., 1992; Bjerner et al., 1986]; Other studies have failed to show clear data on the role of PIIP in pulmonary diseases [Okazaki et al., 1987; Low et al., 1983; Luisetti et al., 1990]. The expression of PIIP turns out to

be highly dependent on the type of lung disease [Okzakai et al., 1987; Low et al., 1983; Cantin et al., 1988]. Interestingly however, within a single disease a large variation in PIIP levels often is also present at the individual level, such as in asbestosis [Cavalleri et al., 1991; Okazaki et al., 1987], interstitial pulmonary fibrosis [Low et al., 1983; Luisetti et al., 1990], sarcoidosis [Pohl et al., 1992; Bjerner et al., 1986; Low et al., 1983; Cantin et al., 1988], farmers lung [Antinnen et al., 1986], or, for instance, in rheumatoid lung disease [Gilligan et al., 1990]. In several studies it has been suggested that serum PIIP measurements can contribute to early diagnosis [Low et al., 1992; Cavalleri et al., 1991; Okazaki et al., 1987; Okazaki et al., 1983]. The only way to determine the role of (serum) PIIP measurements in predicting the development or progression of a disease is through a longitudinal design. However, decent follow-up studies among subjects involved in the original cross-sectional studies [Borm et al., 1992; Borm and Schins, 1993], are mostly lacking.

This article describes the results of a 5-year follow-up study of a cohort of miners designed to test procollagen type III peptide as a biological marker of early diagnosis or progression of Coal Workers Pneumoconiosis (CWP). Previously, we [Janssen et al., 1992] observed that coal miners without radiological evidence of pneumoconiosis had higher PIIP levels than non-dust-exposed control subjects. Second, a decreasing trend in PIIP levels was seen along with the clinical progression of pneumoconiosis (defined by chest X-ray), in line with the fact that type III collagen is mainly formed at early fibrosis. Third, and most important with regard to our follow-up design, a large proportion of subjects with abnormally high ($>\text{mean} + 2\text{SD}$ of non-dust-exposed controls) PIIP levels was observed among healthy miners and miners at early stages of CWP. It was suggested that these individuals had increased fibrotic activity, not detectable in chest X-rays, and as such might be at increased risk to develop or progress in early stage pneumoconiosis. To test this hypothesis, a 5-year follow-up study of the original cohort of coal miners was conducted to measure progression of CWP.

Methods

Subjects and protocol

Miners ($n=156$) from previous studies in 1987 (Fig. 11.1) were contacted and asked to participate in the follow-up study. Chest X-rays were made according to the criteria of the International Labour Organisation [ILO, 1980]. Blood (30 ml) was sampled, treated and stored at -80°C until analysis of serum PIIP levels by radioimmunoassay as described previously [Janssen et al., 1992]. Control levels of PIIP were obtained from 29 age matched, non-dust exposed, male subjects. A complete job history of the subjects was obtained by questionnaire and personal interview. Exclusion criteria for non-dust-exposed controls were (1) working for

more than one year in occupational settings related to chronic dust exposure (mining, cement works, ceramic industry, bakery, etc.) or (2) asthma, (chronic) bronchitis or other chronic diseases of lung, liver and kidney.

Exposure history

Although cases and control miners in our cohorts previously were matched on age and years underground, small differences in exposure might affect conclusions on the use of PIIP as a biological marker. For this reason the occupational history from each subject was gathered. Individual dust exposure was calculated from each individuals' job exposure matrix obtained from a personal interview at follow-up and the medical file. Exposure parameters used for this study were: (1) cumulative dust exposure, (2) cumulative quartz exposure, (3) years underground, (4) years since first exposure, and (5) months retired.

Figure 11.1. Study design and study groups. The shaded area indicates the groups in which serum PIIP has been determined. Serum PIIP levels in 1987 were determined in 73 miners, and in 1992 in 104 miners. The PIIP follow-up subgroup consisted of 51 (out of 73) miners; in 53 (out of 83) miners PIIP levels were measured for the first time in 1992.

ILO classification of chest X-rays and study groups

Chest X-rays taken in 1987 and in 1992 were scored according to the classification rules of the International Labour Organisation [ILO, 1980] by an experienced panel of three physicians. Scoring was done in plenary sessions where consensus had to be reached based on individual scores. Standard ILO chest X-rays were

used for reference to classification. The reading panel consisted of three occupational physicians; two of them were seeing more than 3000 chest X-rays a year, and one was a trained reader but had not been active as such for the last three years. Two of the three readers were also involved in our previous studies. Based on the ILO scores, the dust-exposed cohort was subdivided into 6 groups: 0/0, 0/1, I (=1/0, 1/1, 1/2), II (=2/1, 2/2, 2/3), III (=3/2, 3/3), and IV (=3/+, PMF). Chest X-rays were scored in two plenary sessions. In the first session all X-rays i.e. of 1987 and 1992 were scored randomized blind. In a second session individuals' 1987 and 1992 X-rays photographs were scored pairedwise. The randomized blind reading sessions were used to determine intra-reader variability. However, as a gold standard for 5-year progression or new development of CWP, the outcome from the pairedwise reading session of each individual's chest X-ray of 1987 and 1992 was used. Readers were allowed to consult medical files and additional X-rays from other years to facilitate diagnosis of CWP, if consensus could not be reached unambiguously.

Smoking, medication and medical history

Prior to the medical consultation, an extensive questionnaire was sent to all participants in which questions were asked about medical complaints, medical history, smoking habits and medication. Answers were verified during personal interviews and by comparison with questionnaires from our preceding studies. Smoking amounts were calculated as described elsewhere [Jorna et al., 1994]. Furthermore, subjects were subdivided into current, ever, and never smokers. With respect to medication and medical history, subjects were classified based on the presence or absence of any medication during 3 weeks prior to blood sampling, or according to presence or absence of medical complaints.

Statistical evaluations

Five-year changes in PIIP were tested by paired *t* test. Correlations between PIIP levels of 1987 and 1992 versus exposure, age, smoking, etc., were evaluated by means of multiple linear regression. Unpaired differences between 1987 and 1992 (sub) groups were tested by the Mann Whitney *U* test. Differences between miners without or with CWP and non-dust-exposed controls, and with respect to medication, medical history, smoking were tested by the Kruskal Wallis test and ANOVA. Odds ratio's and confidence intervals were calculated to determine the significance of PIIP levels in predicting 5-year progression of pneumoconiosis. All statistical evaluations were made using STATGRAPHICS (version 6.0).

Results

One hundred and four coal miners participated in our follow-up. Demographic characteristics of these study groups are given in Table 11.1 and Figure 11.1. For 51 miners we had 1987 serum type III procollagen levels from our original study [Janssen et al., 1992]. The other group of 53 miners were also screened in 1987, but in a different survey. When the population parameters of both the original and the follow-up groups were checked, no significant selection on age, smoking habits, medication or years underground was observed. No selection bias with regard to pneumoconiotic stage (i.e., 1987 X-ray) was present.

Table 11.1 Serum procollagen type III peptide (PIIIP) levels, age, exposure, smoking, and the five-year progression of pneumoconiosis in PIIIP subcohort (n=51).

	ILO 1987 = 0/0		ILO 1987 ≥ 0/1		All miners	
Five-year progression	<i>no</i>	<i>yes</i>	<i>no</i>	<i>yes</i>	<i>no</i>	<i>yes</i>
n	39	2	4	6	43	8
1987 PIIIP (ng/ml)	61.26 (2.3)	70.45 (11.1)	63.93 (3.0)	52.02 (4.6)	61.51 (2.1)	56.63 (5.0)
age (years)	47.2 (0.7)	47.5 (5.5)	49.5 (2.0)	54.0 (1.2)	47.4 (0.7)	52.4 (1.7)*
exposure:						
cum. dust (g·h·m ⁻³)	91.6 (9.4)	155.0 (45.0)	143.0 (5.1)	110.0 (31.2)	96.4 (8.8)	121.3 (25.4)
years underground	22.5 (0.7)	26.0 (6.0)	26.5 (1.6)	27.0 (1.8)	22.8 (0.6)	26.8 (2.0)#
years since first exposure	29.5 (0.9)	29.5 (7.5)	33.3 (2.1)	35.2 (1.5)	29.9 (0.8)	33.8 (2.0)
smoking (packyears)	124.2 (20.0)	134.0 (56.0)	148.0 (69.6)	200.8 (55.0)	126.5 (18.9)	184.1 (43.0)

Values are mean and standard error. ILO = 1987 chest radiograph according to ILO criteria.

* $p=0.012$, and # $p=0.035$, significantly different from non-progressed (in whole miners group)

Retirement

During the follow-up period all miners had left active mining. Occupational exposure had ended for at least 4 years in half of our miners group and for at least 2 years in more than 85% of the miners. No selective loss to follow-up had taken place with regard to retirement (Figure 11.2). The average age of retirement in this group was 44.7 ± 5.1 (mean $\pm$ SD).

Figure 11.2. Retirement in original cohort (n=156) and follow-up group (n=104) during the five year follow-up interval.

PIIIP measurements of 1987 and 1992

Paired testing of the PIIIP data of 1987 and 1992 in 51 coal miners revealed a significant difference ($p < .001$) between serum PIIIP levels in these two years. Also the mean, mode, minimum and maximum of all 1992 values (n=104) were lower than all 1987 values (n=71). In Figure 11.3A, serum PIIIP levels of the various groups are compared, as determined in 1987 and 1992. In 1992, mean PIIIP levels of miners without radiological evidence of CWP, and those of miners of group I and group II were significantly lower than 1987 ($p < .01$). Significantly lower PIIIP levels were also observed for the non-dust-exposed control group in 1992 ($p < .01$, Figure 11.3A). Since these differences might be a consequence of some major modifications in the radioimmunoassay of the procollagen peptides, serum PIIIP values were also calculated as a percentage of the non-dust-exposed control levels (Figure 11.3B). After this correction, differences between serum PIIIP levels within the different categories remained apparent, although they were significant only for the control miners group.

Figure 11.3. Comparison of serum procollagen type III peptide (PIIIP) between the 1987 and 1992 study groups. On the horizontal axis subgroups are 0/0, 0/1, group I (1/0,1/1,1/2), group II (2/1, 2/2, 2/3), group III (3/2, 3/3), group IV (progressive massive fibrosis), and non-dust-exposed controls (ndc).

Figure A represents absolute values (ng/ml); in figure B values are expressed as a percentage of the non-dust-exposed controls. Bars represent mean and standard deviations. The asterisk denotes a significant difference ($p < 0.05$) between subgroups of 1987 (shaded bars) and 1992 (open bars).

Unlike in our previous findings, PIIIP levels were not significantly increased in coal miners versus their non-dust-exposed controls. Furthermore, the downward trend of PIIIP levels versus pneumoconiotic stage (defined by chest X-ray) was not observed at present (figure 11.3A and 11.3B). Moreover, all subjects now have normal serum PIIIP levels (<mean +2SD of the non-dust-exposed control group). Evidently a poor relation is present between serum PIIIP levels at the two time points measured, demonstrated by a poor correlation in the total

group (n=51) and a significant negative correlation between 1987 and 1992 levels in the CWP group (see table 11.2).

Table 11.2 Correlation of PIIP-data 1987 versus 1992 (n=51).

group	n	r	p-value
all miners	(51)	0.12	0.39
ILO 1987 = 0/0(41)		0.21	0.20
ILO 1987 $\geq$ 0/1(10)		-0.69	0.02

r = Coefficient of linear correlation;

ILO 1987 = chest radiograph according to ILO criteria.

Serum PIIP and progression of CWP

In our PIIP follow-up cohort (n=51), no correlation was observed between PIIP levels and smoking (packyears), age, or any parameter of exposure. Moreover, serum PIIP levels were not significantly different with respect to medication, medical history, or past and current smoking habits (n=104). Interestingly, a significant correlation was observed between serum PIIP and weight ($r=.26$, $n=104$, $p<.01$), but none of the above parameters were correlated to serum PIIP upon correction for body weight (ng/mL kg^{-1}).

Despite retirement, a significant proportion (16%) of this cohort showed progression of CWP during the 5-year follow-up period. Four out of 80 healthy coal miners contracted CWP, and 13 out of 25 cases CWP did progress. In the original cohort of miners involved in the procollagen study of 1987, 2 miners newly developed pneumoconiosis and in 6 out of 10 subjects already existing pneumoconiosis progressed. However, none of these subjects had abnormal levels of procollagen (ng/mL , ng/mL kg^{-1}) in 1987 or in 1992. Furthermore, in the PIIP follow-up cohort (n=51), 5-year changes in pneumoconiosis were not related to original procollagen levels measured in 1987 (Table 11.1). A multiple regression of serum PIIP of 1987 versus 1992, exposure and age revealed no significant correlations and therefore excludes bias from these parameters on serum PIIP levels. There was also no difference in exposure, age or smoking habits between miners without and miners with progression of coal workers pneumoconiosis (Table 11.1).

Discussion

Nowadays, coal workers' pneumoconiosis (CWP) has become a disease of delayed onset owing to a more efficient technological and medical prevention. This epidemiological evolution necessitates postprofessional medical follow-up of coal miners [François et al., 1988]. In the UK simple pneumoconiosis has become more frequent among men who have left industry than in the active work force [Soutar et al., 1986]. In the Lorraine mining region in France 82% of the 1986 incidence of CWP was observed in retired coal workers [Mahieu, 1990]. Complementary to these epidemiological findings, comprehensive biological explanations for the individual development and progression in CWP still need to be elucidated. Since this entire cohort had retired from active mining since first surveyed, our data on progression of CWP related to serum PIIP might as such be useful in the postprofessional survey of coal miners. Despite retirement, a significant proportion (16%) of the cohort showed progression of CWP. However, all miners of this cohort previously had been selected on long-term and high occupational exposure, and as such do not reflect progression rate in the general population of retired miners in the Kempen mining region in Belgium.

With respect to the determination of serum PIIP, our previous findings in active miners [Janssen et al., 1992] were not confirmed, since this time: (1) no relationship between CWP stage and PIIP levels were observed and (2) mean serum PIIP levels were not significantly elevated in (retired) coal miners versus their non-dust-exposed reference group. Moreover, (3) at present no individuals showing abnormal high levels of serum PIIP, i.e., out of the normal range (2 SD above mean) of the non-dust-exposed controls, were seen. One might attribute this to the retirement of the miners and the cessation of dust exposure. However, since no relation between the decrease in serum PIIP and period of retirement was present (data not shown), this is unlikely to be a plausible explanation. Differences between the two non-dust-exposed control groups in both studies only strengthen the discrepancies: Unlike in our previous study, in which anonymous subjects were randomly selected from the University Hospital blood bank, in the present study we excluded subjects with occupational dust exposure and/or medical treatment and the presence of fibrotic lesions.

This 5-year follow-up study does not forward the use of serum PIIP measurement to predict either development or progression of CWP. The relative risk for progression of a miner showing an abnormal PIIP level in 1987, as well as the calculated odds ratio of the 1992 PIIP levels for progressed subjects versus non-progressed subjects, are both below 1, and rather suggest a tendency toward increased risk for coal miners with normal PIIP levels. Our results are in contrast to a five year follow-up study in sarcoidosis patients [Pohl et al., 1992], where serum PIIP levels were found to be a very good predictor of disease

progression. One should keep in mind that sarcoidosis, in contrast to CWP, is characterized by a striking interstitial fibrosis and has a completely different origin [Snyder, 1988; Luisetti et al., 1990]. However, in an other study in sarcoidosis patients, serum PIIP failed to predict disease prognosis [Luisetti et al., 1990]. We suggest that the types of disease and variations in fibrotic activity within one type of lung disease, reflecting moments of increased inflammation, tissue damage, wound healing, and collagen turnover, affect serum PIIP levels. Therefore, serum determinations do not necessarily reflect the stage of disease. Perhaps serum PIIP is a marker of increased fibrotic activity, but then in this cohort the individuals were not at higher risk for progression of CWP. Unfortunately, this cohort comprised only retired miners and therefore discrimination between cessation of exposure and time variation as the two possible causes of the lower serum levels in 1992 was not possible. Data on both the effect of exposure and time variation in serum PIIP are scarce and therefore application of this measurement as a diagnostic marker in postoccupational screening in CWP and other fibrotic lung disorders should be done with the utmost care.

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Chapter 12

EVALUATION OF SERUM TYPE III PROCOLLAGEN PEPTIDE AS AN EXPOSURE MARKER IN RETIRED COAL WORKERS.

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Abstract

Serum type III procollagen peptide (PIIIP), a degradation product of the type III collagen precursor has been put forward as an exposure marker for mineral dust. We evaluated PIIIP levels as a marker of exposure to and effects of coal dust in retired coal miners (n=104). To this end: (a) the individual cumulative dust exposure was calculated from job-exposure matrices, and (b) in addition to a routine chest radiograph (CR) of all miners according to the criteria of the International Labour Organisation (ILO), a subgroup (n=46) was screened by high resolution computed tomography (HRCT). Profusion score (CR and HRCT) tended to increase with cumulative dust exposure, even in the absence of CR evidence for pneumoconiosis (i.e. CR≤0/1, n=35). In contrast to our previous findings in active miners, PIIIP levels were not increased in miners compared with non dust-exposed controls (n=29), and no differences were observed between miners without (ILO = 0/0) and miners with coal workers' pneumoconiosis (CWP; ILO ≥0/1). No trend in PIIIP versus pneumoconiosis stage was present, either by CR or by the more sensitive HRCT score. PIIIP was also unrelated to any lung function parameter (FEV₁, FVC, impedance, diffusion capacity). Age, medication, medical history and smoking habits had no significant effect on PIIIP levels. In the miners with CWP (i.e. ILO>0/0, n=28) a significant negative correlation was present between PIIIP values and (log) cumulative dust exposure. This decrease in serum PIIIP levels with increasing cumulative exposure may be due to chronic adaptive changes in type III collagen deposition and/or breakdown. Other relations between exposure and PIIIP were not observed. In conclusion, the present findings do not support the use of serum type III procollagen peptide as a marker of exposure to and (early) interstitial or respiratory effects of coal dust.

Introduction

Serum or bronchoalveolar lavage levels of the type III procollagen peptide (PIIIP) have been studied in relation to several lung diseases, including asbestosis [Okazaki et al. 1987; Cavalleri et al. 1988; Cavalleri et al. 1991], idiopathic pulmonary fibrosis [Low et al. 1983; Cantin et al. 1988; Luisetti and Bulgheroni 1990], sarcoidosis [Bjerner et al. 1986; Pohl et al. 1992], farmer's lung [Anttinen et al. 1986], and rheumatoid lung disease [Gilligan et al. 1990]. In spite of major differences in the morphology, pathological observations, and clinical course of many of these diseases, the levels of serum (or bronchoalveolar lavage) PIIIP are believed to reflect the current activity of an on-going fibrotic disease process, characterized by the deposition of collagen fibrils at fibrotic sites [Gilligan et al. 1990; Pohl et al. 1992; Low et al. 1992].

Previously we found [Janssen et al. 1992] that serum PIIIP levels were increased in coal miners as compared with non dust-exposed controls and we observed a relationship between PIIIP level and stage of coal workers' pneumoconiosis (CWP) as defined by chest radiograph (CR) according to the criteria of the International Labour Organization [ILO 1980]. We, therefore suggested that routine measurements of PIIIP in serum could be applied for early diagnosis of CWP [Borm et al. 1992].

Based on highly significant correlations repeatedly observed between asbestos exposure and serum PIIIP in asbestos exposed workers [Cavalleri et al. 1988, 1991], PIIIP measurements have recently been forwarded as a valid tool in occupational monitoring of subjects with mineral dust exposure [Becklake et al. 1993]. In order to evaluate PIIIP as a marker of exposure to and effects [Schulte and Perera 1993] of coal dust, the original cohort of coal miners (meanwhile all retired) were recruited for a five-year follow-up study. In all miners serum PIIIP was determined, new CRs were obtained, lung function was determined by spirometry and impedance measurement, individual cumulative exposure was estimated and a subgroup was screened using high resolution computed tomography (HRCT). This follow-up showed that a significant proportion of our miners, all previously heavily exposed, progressed in CWP in spite of retirement [Schins et al. 1994]. Prospective analysis of the data however, showed that the initial PIIIP measurements were not predictive of the 5-year development or progression of pneumoconiosis [Schins and Borm, 1994]. In the present paper serum PIIIP is evaluated as an exposure marker to coal mine dust and is also compared to several indices of interstitial and respiratory effects of coal dust.

Subjects and methods

Subjects

Retired coal miners (n=104) from a combined cohort of three previous studies [Borm et al. 1988; Engelen et al. 1990; Janssen et al. 1992] among miners from the Belgian coal mining company "Kempense Steenkoolmijnen" participated. Written informed consent was obtained from each worker and after completion of a questionnaire (see below), blood (10 ml) was sampled, lung function was determined (spirometry, impedance) and CRs were made. Blood samples were obtained and treated as described previously [Engelen et al. 1990] and serum was stored at -80°C until analysis of PIIP. Serum levels of PIIP were determined by the radioimmunoassay method originally developed by Rhode and colleagues, as described previously [Rhode et al. 1979; Janssen et al. 1992]. PIIP levels were determined in all miners (n=104) and in 29 male, age matched, non dust-exposed controls. On a voluntary basis, HRCT slices were made in a subgroup of 46 miners. Smoking habits, medication and medical history were derived from each subject as described previously [Jorna et al. 1994a; Schins and Borm 1994].

CR and HRCT

CR were taken and scored according to the criteria of the ILO [1980]. Scoring was done as described previously [Schins and Borm 1994; Schins et al. 1994]. Miners with an ILO profusion score 0/0 were considered to be "healthy coal workers" (HCWs), and miners with an ILO category $\geq 0/1$ as miners with CWP.

HRCT slices were made and scored as described previously in a subgroup of 46 miners [Lamers et al. 1994]. Since collagen type III is believed to be mainly involved at early stages of active interstitial fibrosis [Bateman et al. 1981] we focused our attention on the relationship between serum PIIP levels and HRCT in miners without the classic-diagnostic evidence for CWP. Therefore, the HRCT subgroup was divided on the basis of their CR score into miners without CWP or with an early/doubtful stage of CWP (i.e. CR=0/0 and 0/1) versus miners with definite evidence for CWP (i.e. CR $\geq 1/0$).

Exposure

Considering the aim of the study, much emphasis was placed on a good estimation of exposure and several parameters were used, i.e. (a) time worked underground, (b) time since first occupational exposure, (c) time since last occupational exposure, (d) cumulative exposure to respirable coal dust and quartz content. Years exposed, time since first exposure and time elapsed since last occupational exposure were determined from the medical/personel file and a personal interview at follow-up.

Exposure years were determined from the complete job history obtained from each subject and defined as the total number of years in which a subject worked underground in a coal mine. Long term absenteeism (e.g. due to disease or special leave) as well as interim job changes were taken into account. Years since first exposure were defined as the interval between the year in which a miner started working underground and the time of our study. Time from last exposure was the interval between retirement and our follow-up in months. Job exposure matrices were used to estimate each individual's (respirable) cumulative dust and quartz exposure in gram x hours per cubic meter of sampled air, as described elsewhere [Jorna et al. 1994a].

Lung function

Lung function (spirometry, impedance) was performed in all coal miners as described previously [Jorna et al. 1994a]. Flow-volume parameters used in this study are FEV₁ (forced expiratory volume during first second), FVC (forced vital capacity), and the ratio FEV₁/FVC. Airflow limitation was defined as an FEV₁ less than 80% predicted value. Impedance parameters used are the resistance at 8 and at 28 Hz (R8Hz, R28Hz) and the resonant frequency (f_0). In the subgroup screened for HRCT (n=46), spirometry was repeated, and supplemented with TLC (total lung capacity) and DL_{CO} (diffusion capacity, using carbon monoxide transfer factor), as described elsewhere [Lamers et al. 1994].

Statistical evaluations

Statistical evaluation was made using STATGRAPHICS version 6. Correlations between PIIP levels and exposure, age, smoking and lung function parameters were evaluated by means of (multiple) linear regression. Differences between miners without or with CWP and non-dust-exposed, and with respect to medication, medical history, and smoking were tested by the Mann-Whitney U-test and the Student-t test (ANOVA). Trend analysis between pneumoconiosis stage (CR, HRCT) and PIIP, exposure or other variables were performed by means of the Spearman's rank correlation procedure. For this purpose, profusion scores were converted to a scale from 0 to 11 for CR and 0 to 80 for HRCT.

Results

Radiology outcome

The calculated cumulative exposure was bimodally distributed, due to significantly higher exposure among miners with CWP ($p < 0.01$, Mann-Whitney test), as shown in figure 12.1. The outcome of radiology was significantly related to cumulative exposure, whether based on CR (n=104, $p < 0.001$; Spearman's

$\rho=0.34$) or on HRCT ($n=46$, $p<0.005$; $\rho=0.44$) analysis. Years underground also correlated with CR-scored pneumoconiosis ($\rho=0.29$, $p<0.005$, $n=104$), and to a lesser degree with HRCT-scored pneumoconiosis ($r=0.36$, $p<0.02$, $n=46$). Interestingly, a correlation was also present between years since first exposure and HRCT-defined lesions ($r=0.38$, $p<0.02$, $n=46$), while a similar relation for years since first exposure and CR-scored pneumoconiosis was not present.

Figure 12.1 Frequency distribution of cumulative dust exposure in reference miners (HC) and miners with CWP. The cumulative exposure was normally distributed in the miners with CWP ($CR\geq 0/1$, $n=28$, $c^2=1.47$; $p>0.2$), but not in the miners without CWP ($CR<0/1$, $n=76$, $c^2=16.7$; $p<0.01$).

Table 12.1 compares the classification of miners according to CR versus HRCT. Although visual score of HRCT correlated significantly with CR-score ($\rho=0.53$, $n=46$, $p<0.001$), the data also demonstrate that small opacities are better visible in HRCT slices. About 50% of the miners (16 out of 35) with CR score 0/0 or 0/1 had a negligible number of lesions (score <5) on their HRCT slices. However, the other miners without CR evidence for pneumoconiosis had a large number of small opacities only detectable by HRCT. These observations are in line with findings of others [Bégin et al. 1990; Remi-Jardin et al. 1990; Vermeire 1993] and clearly demonstrate that HRCT permits a gradation of (mild) pneumoconiosis in "CR-normal" miners, as a consequence of the higher resolution of the method. In subjects with CR evidence for pneumoconiosis, CR and HRCT score are usually well in line.

Evaluation of serum PIIP as effect or exposure marker

Serum PIIP values ranged from 18.3 to 81.8 ng/ml, with a mean ($\pm$ standard error) of 36.0 ± 0.72 ng/ml ($n=133$; all miners plus non dust-exposed controls). Medication, medical history or past and current smoking habits had no significant effect on PIIP levels and they were also not associated with age or any parameter of exposure. Furthermore, no relation between PIIP and time of retirement was apparent. Serum PIIP levels were not significantly different between miners without pneumoconiosis ($n=76$; 35.1 ± 0.9 ng/ml) and those with pneumoconiosis ($n=28$; 36.2 ± 1.2 ng/ml), nor was there any difference from the non-dust-exposed controls ($n=29$; 38.6 ± 2.0 ng/ml). No significant correlations were found between serum PIIP and any of the lung function parameters or radiographic score either by CR ($n=104$, $p>0.5$) or HRCT ($n=46$, $p>0.9$). Furthermore, mean ($\pm$ standard error) levels of serum type III procollagen were not different for miners without airflow limitation ($n=92$, 35.1 ± 0.8) and miners with airflow limitation ($n=10$, 36.1 ± 2.9).

Subsequently, several indices of both interstitial (CR, HRCT, DL_{CO}) and respiratory (spirometry, impedance) effects of coal dust exposure were related to serum type III procollagen (Table 12.2). Using the mean serum PIIP value of all miners as a cut-off point, this operation shows that PIIP does not discriminate between any of the parameters, including cumulative exposure. Moreover, linear correlations between serum PIIP and exposure, age or smoking amounts (Table 12.3) showed that serum PIIP levels were not related to years underground or to years since first exposure. Cumulative dust exposure was not related to serum PIIP in miners without CWP, but interestingly in the CWP group a significant negative correlation was present between cumulative dust exposure and PIIP values (Figure 12.2).

Table 12.1 Pneumoconiosis stage by chest radiograph (CR) and HRCT score ($n=46$).

HRCT#		<5	5-10	10-15	15-20	20-30	≥ 30
CR*	0/0	14	9	2	5	2	1
	0/1	2					
	1/0, 1/1, 1/2		1	1		1	
	2/1, 2/2, 2/3				1	3	
	3/2, 3/3, 3/+					1	3

#, * See materials and methods section for details of classification

Table 12.2 Demographic characteristics, lung function and radiology in retired miners when classified according to their serum PIIIP levels.

Parameter**	Unit	PIIIP * < mean	PIIIP ≥ mean
		(n=55)	(n=49)
Age	(y)	48.1 ± 0.7	48.7 ± 0.8
Cumulative dust	(gh/m3)	97.6 ± 7.2	105.9 ± 8.8
Smoking	(packyears)	127.3 ± 14.1	115.3 ± 17.6
FEV ₁	(L)	3.49 ± 0.10	3.64 ± 0.10
FEV ₁	(%)	102.1 ± 2.4	105.0 ± 2.3
FEV ₁ < 80% predicted		5	5
FVC	(L)	4.62 ± 0.11	4.70 ± 0.12
FVC	(%)	110.5 ± 2.1	111.1 ± 2.0
FEV ₁ /FVC		0.75 ± 0.01	0.77 ± 0.01
R8Hz	(hPa•s/L)	2.66 ± 0.13	2.51 ± 0.16
R28Hz	(hPa•s/L)	2.46 ± 0.09	2.54 ± 0.11
f ₀	(Hz)	11.0 ± 0.6	10.0 ± 0.5
CR score	0/0	4	36
	0/1	3	4
	1/0, 1/1, 1/2	1	4
	2/1, 2/2, 2/3	6	2
	3/2, 3/3, 3/+	5	3
		(n=23)	(n=23)
TLC	(%)	102.0 ± 2.5	99.5 ± 2.5
DL _{CO}	(%)	110.2 ± 5.4	116.7 ± 5.5
HRCT score	<5	8	8
	5-10	5	5
	10-15	2	1
	15-20	3	3
	>20	5	6

* The mean PIIIP value (35.3 ng/ml) of all miners was used as a cut-off point.

** FEV₁: Forced Expiratory Volume in first second; FVC: Forced Vital Capacity; R8Hz: Resistance at 8Hz; R28Hz: Resistance at 28Hz; f₀: Resonant Frequency; TLC: Total Lung Capacity; DL_{CO}: Diffusion capacity; See methods section for details on CR (chest radiography), HRCT (High Resolution Computed Tomography), and exposure assessment.

*** No statistically significant differences (p<0.05) were detected using either student t-test, Mann-Whitney U-test or χ^2 -test (proportions only).

Table 12.3 Linear correlations between serum PIIIP levels and exposure, age, and smoking

	group ^a	group size (n)	correlation coefficient
cumulative dust (ghm ⁻³)	HCW	(76)	0.09
	CWP	(28)	-0.46*
	HCW and CWP	(104)	0.01
cumulative quartz (mghm ⁻³)	HCW	(76)	0.09
	CWP	(28)	-0.43*
	HCW and CWP	(104)	0.02
years underground	HCW	(76)	0.05
	CWP	(28)	-0.09
	HCW and CWP	(104)	0.03
years since first exposure	HCW	(76)	-0.21
	CWP	(28)	0.12
	HCW and CWP	(104)	-0.19
age (years)	HCW	(76)	-0.16
	CWP	(28)	0.04
	NDC	(29)	-0.30
	HCW and CWP	(104)	-0.13
smoking (pack years)	HCW	(76)	-0.05
	CWP	(28)	-0.19
	NDC	(29)	-0.08
	HCW and CWP	(104)	-0.07

^a HCW, CR=0/0; CWP, CR≥0/0; NDC, non dust-exposed control group; * significant at p=0.05

Discussion

In the present study we evaluated serum PIIIP levels of coal miners in relation to occupational exposure to coal dust, radiographic findings, and lung function in order to evaluate the use of serum PIIIP as an exposure and effect marker [Borm et al. 1992; Borm 1994]. Previously we found that PIIIP levels in serum were increased in active coal miners (n=73) as compared with non-dust exposed controls (n=19), and a downward trend was observed in PIIIP levels versus pneumoconiosis stage as classified by CR [Janssen et al. 1992]. In the current study among retired miners, however, these findings were not confirmed, and no relationship between serum PIIIP and CR [Schins and Borm 1994], or between PIIIP levels and HRCT readings (n=46) was found. In addition, serum PIIIP levels were not related to the outcome of flow-volume curves, diffusion capacity or impedance measurements.

Figure 12.2

The relation between serum PIIP and cumulative dust exposure in miners with CWP ($n=28$), illustrated as linear correlation in panel A ($r=-0.46$, $p<0.05$). This correlation improves considerably if exposure is expressed on a logarithmic scale, shown in panel B ($r=-0.55$, $p<0.005$).

In contrast to our findings with respect to pulmonary function, Bjermer *et al.* reported negative correlations between PIIP in bronchoalveolar lavage and lung function tests (i.e. VC, FEV₁, DLCO) in sarcoidosis patients [Bjermer *et al.* 1986]. Similarly, Cavalleri *et al.* [1991] have reported significantly higher levels of serum PIIP in asbestos exposed workers with both FVC and FEV₁ below the 100% predicted value compared to workers with values equal to or exceeding this cut-off level. No such relationship between lung function and serum PIIP levels was observed in our study, using either 100% or 80% as a cut-off value for FEV₁ and FVC. A reverse analysis, using high and low serum PIIP as a standard (Table 12.2) failed to reveal any significant difference in lung function, or any other effect, between the two groups.

Our data are in line with the outcome of a workshop on "health risks from exposure to mineral fibres" recently held in Japan [Becklake et al. 1993], where the diagnostic role of serum PIIIP measurements was still considered limited. However, the working group concluded that PIIIP measurements in serum "could be used to consider research questions related to biological monitoring strategy". This statement was largely backed up by correlations between years exposed to asbestos and PIIIP levels in a group of six individuals [Cavalleri et al. 1988], and more recently in 21 subjects based on calculated asbestos exposure [Cavalleri et al. 1991]. However, our study (n=104 coal miners) did not reveal any clear positive dose-response between serum PIIIP levels and exposure, whether defined as cumulative dust exposure, years underground or years since first exposure. Moreover, a retrospective analysis revealed no significant correlation of PIIIP measured in 1987 and cumulative dust exposure in a subgroup of miners when still active (n=51, $r=0.13$). In addition, years underground, the exposure parameter known for all miners (n=73) in the previous study, was not related to serum PIIIP levels determined at that time [Janssen et al. 1992].

Our data certainly do not suggest that exposure is the only determinant of the observed PIIIP levels, especially in the subgroup of miners with CWP. In this group a significant negative correlation was observed between PIIIP values and (log) cumulative dust exposure. Multiple regression, however, did not reveal other variables (i.e. age, smoking, profusion score, lung function, retirement) involved in the expression of PIIIP in pneumoconiosis. Interestingly, we also observed an inverse relation between cumulative respirable dust exposure and serum PIIIP in potato sorters [Jorna et al. 1994b] in the absence of radiological evidence for pneumoconiosis. In contrast to the miners in this study, these were all active workers with current exposure to mainly non-crystalline silica. Again no relation between PIIIP and lung function was apparent.

We can only speculate on the underlying reasons for the discrepancies among studies on the relation between PIIIP and respiratory or interstitial effects, but suggest that differences in total cumulative exposure, frequency and intensity of exposure, lung retention time, and mineral characteristics (silica, coal dust, asbestos) are involved. The relation observed between PIIIP and cumulative coal dust exposure, but interestingly not between PIIIP and years underground, suggests a significant effect of intensity and frequency of exposure when compared to duration. Secondly, the time- and clinical course in collagen type III deposition and degradation may also be involved. Both animal and human data have demonstrated that the early increase in type III collagen levels observed in active fibrotic parts of the lung is followed by a significant reduction of type III collagen involvement in favour of other collagen types [Bateman et al. 1981; Laurent and McAnulty 1983; Arden and Adamson 1992]. Therefore, persistent fibrosis might be characterized by lower PIIIP levels, which is supported by

observations in postmortem biopsies [Seyer et al. 1976; Madri and Furthmayr 1980]. In conclusion, our findings possibly reflect chronic adaptive changes in type III collagen deposition or breakdown, that seem to continue even several years after the cessation of occupational exposure. Whether these may be caused solely by repetitive high peak exposures during occupation [Seaton et al. 1981], or are a consequence merely of the total dust load present in the fibrotic lung remains a question open for further research.

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Chapter 13

SUMMARY AND GENERAL DISCUSSION

Mineral dust induced lung disorders are among the most widespread and most prevalent occupational lung diseases in the world [Mossman et al., 1990; Cullen et al., 1990; Oxman et al., 1993; Rom, 1992; Meredith and McDonald, 1994]. Most likely as a consequence of the specific toxicokinetic properties of mineral dusts (e.g. durability, particle overload, see chapter 1), disease may even progress after exposure has ended [Jones et al., 1989; Cullen et al., 1990]. In spite of tremendous efforts to reduce exposure, mineral dust induced lung diseases, including pneumoconiosis have not yet ceased to exist, and regularly new cases are being reported both among well established risk groups and less well expected environments [Kauffman et al., 1982; Landrigan et al., 1987; Baris et al., 1988; Cullen et al., 1990; Nemery et al., 1992; Oxman et al., 1993]. Unfortunately, medical treatment of chronic lung disorders such as pulmonary fibrosis is still limited in its efficiency to date [DeRemee, 1994; Goldstein and Fine, 1995; Hunninghake and Kalica, 1995]

As classical occupational epidemiological studies yield less clear cut results due to lower exposure levels (limit values), utilization of biomarkers may be a valuable contribution to epidemiologic research. This also holds for their application to workplace monitoring, medical screening and post-exposure surveillance. Additionally, during the search for these biomarkers, new insights in pathogenic mechanisms may be generated, to identify those at risk or to develop new methods of medical cure and care in those who have become diseased [Schulte et al., 1993; Weill, 1994].

In the present thesis several biological markers of mineral dust induced respiratory disorders were evaluated, by recruitment of coal miners originally involved in several cross-sectional surveys. In other words, molecular epidemiology studies in coal workers linking the "black seam" with "black lungs" have been used to further open the "black box" between mineral dust exposure and resultant chronic lung disorders. This concept is schematically shown in figure 13.1. In the present thesis we focussed on the two mostly appreciated biological mechanisms known to occur in response to mineral dust inhalation, i.e. (1) the generation of reactive oxygen species (ROS) and the related antioxidant defenses, and (2) the involvement of cytokine networks between and among inflammatory cells and structural cells in the lung (see figure 13.1). Furthermore, collagen deposition, recognised as the hallmark of pulmonary fibrosis, was evaluated.

At the time of follow-up all miners had retired from active mining as a result of the closing of the "Kempen" coal mines. Nevertheless in a significant proportion

Figure 13.1 Framework of events (left) in mineral dust exposure and resultant lung disease (middle) and biomarkers evaluated (right). The presented framework (described in chapter 1) has been applied to evaluate a set of biological markers that have been forwarded from the biological processes considered to play an important role in mineral dust induced lung disorders (chapters 2 and 3). The markers that have been evaluated in view of this thesis are depicted in the right panel: Peripheral blood parameters (dark box) are related to parameters of mineral dust exposure (upper open box), of lung disorders (middle open box), and of clinical progression of these disorders (lower open box).

of the miners five-year deterioration of chest radiography determined pneumoconiosis lesions was observed, including identification of some “new” CWP cases (chapter 8 and 11). Estimation of the cumulative exposure to respirable dust by means of a job-exposure matrix approach, showed that although initially the miners had been matched on years of underground employment, actual exposure estimates varied considerably: cumulative dust exposure data had an approximately fourfold variation compared to years underground (chapter 12). Paradoxically, it was this variation which was used to validate markers as “exposure markers”, and could explain why few biological events were significantly related to exposure. Interestingly, a good correlation between cumulative dust exposure and conventional chest radiograph- as well as HRCT score was found (chapter 12), and opposite to other exposure parameters, the cumulative dust exposure was clearly related to the presence as well as the progression of CWP (chapter 8). Inclusion of HRCT analysis in a subgroup of miners resulted in a fine-tuning of early stage pneumoconiosis (chapter 12), while validated questionnaires, lung function measurements as well as retrospective analysis of longitudinal lung function decline allowed to relate blood markers to obstructive lung disorders (chapter 3). The significance of the biomarkers evaluated in this thesis (see figure 13.1) is discussed below, in line with the structure of the introduction (chapter 3) based on their biological background.

OXIDATIVE DAMAGE AND ANTIOXIDANT STATUS

The increased ratio of 8-oxodG/dG in lymphocyte DNA of the miners (chapter 6) suggests that oxidative stress responses occur outside the lung. This finding is in line with previous findings from our and other labs [Engelen et al., 1990; Nadif-Perrin et al., 1995]. They also fit with the results described in chapters 5 and 7, showing that serum and red cell antioxidants are affected in subjects chronically exposed to coal dust. Interestingly, these effects were seen in the absence of acute exposure, since all miners were retired for considerable time at follow-up. Furthermore, these effects are relatively independent of the presence of fibrosis, since increased 8-oxodG levels were already found in those exposed but without CWP. However, from these data one cannot conclude in favour of the suggested direct carcinogenic action of mineral dusts (e.g. silica) as compared to the suggested indirect action via (a) mechanisms occurring in the presence of fibrosis, or via (b) dust acting as carcinogen carriers (e.g. PAH) [Goldsmith et al., 1986; McDonald, 1989]. A possible link between the observed damage in the DNA of lymphocytes and target DNA (e.g. bronchial epithelium) remains to be evaluated, and therefore it is not clear whether this oxidative stress response is related to carcinogenic risk. Nevertheless, our observations are in line with the

data of Dalal [Dalal et al., 1991] who showed that increased number of stable radicals (determined by ESR) were found in miners compared to controls. The increased number of such stable radicals in lymph nodes of the lung may thus be one explanation for the observed DNA damage in lymphocytes.

Total serum antioxidant capacity, as evaluated by TRAP, was not different between miners with or without CWP, and not related to erythrocyte GSH content (chapter 5). Interestingly however, both TRAP and GSH tended to be higher in those with previous development of CWP (retrospective analysis), while initial GSH, as well as Vitamin E serum levels were lower in subjects with later development of pneumoconiosis (prospective analysis) (chapter 7). It should be realized however, that GSH was determined by different methods in the original study and during follow-up, and Vitamin E is considered to represent only a fraction of the total antioxidant capacity in the serum. In those miners with progression of already existing CWP, also red cell superoxide dismutase activities were higher. Since *in vitro* and *in vivo* observations have shown that increased MnSOD expression is an indicator of oxidative stress [Janssen et al., 1994a], one could suggest higher stress in those with progression of CWP as a consequence of increased inflammatory reactions.

When evaluating their applicability as exposure or effect markers it appeared that serum or red cell antioxidants were not related to (cumulative dust) exposure or to severity (chest radiography) of CWP. However, TRAP tended to go down with increased profusion and in those without progression (inactive fibrosis) TRAP was inversely related to cumulative dust exposure (chapter 5). Furthermore red cell antioxidant enzymes (GST, SOD, Catalase) were retrospectively related to lung function decline (chapter 7), and GSH levels were significantly reduced in chronic bronchitis. A particular role may be in store for erythrocyte GST, as reduced activity of this enzyme denominates increased risk for progression of pneumoconiosis, while high activity was associated to increased lung function loss (chapter 7). In conclusion, these data indicate the presence of oxidative stress in subjects chronically exposed to mineral dust (even after mineral dust exposure has ended for considerable time), as well as the significance of these responses in the development or progression of mineral dust induced disorders, including pneumoconiosis and obstructive lung disease. Antioxidant activities in red cells and serum, particularly if they are combined in a multi-marker evaluation, could be implemented in occupational screening as well as in post-occupational surveillance.

CYTOKINE NETWORKS

Following the observation that *ex vivo* stimulated release of TNF was increased in active miners [Borm et al., 1988], the crucial involvement of TNF in mineral

dust induced fibrosis was demonstrated *in vivo* by Piguet and colleagues in a mouse model of silica induced fibrosis [1990]. In the present study, support for the crucial role of TNF in coal dust induced fibrosis, was obtained in various ways: (1) The repeated cross-sectional analysis (chapter 8) confirmed previous observations [Borm et al., 1988]. (2) The prospective design showed that high monocyte TNF release was clearly related to the risk for progression of CWP (chapter 8). (3) Plasma soluble receptors of TNF (i.e. sTNF-R55 and -R75) were elevated in coal workers compared to non-exposed individuals (chapter 9). The significance of the soluble TNF receptors as susceptibility markers in pneumoconiosis still needs to be evaluated, taking into account a possible confounding effect of medication on plasma sTNF-R55 levels (chapter 9). Interestingly, both erythrocyte SOD activity and monocyte TNF release were risk factors for progression of CWP (chapters 7 and 8). No correlation was found between both parameters at follow-up, although TNF is known as a powerful stimulus of mRNA expression of MnSOD in various target cells [Wong and Goeddel., 1988]. Monocyte release of TNF was not related to any parameter of exposure, both in the original [Borm et al., 1988] and the follow-up study (chapter 8). Significant correlations between TNF release measured in the original study and at follow-up, were found in response to coal dust or 3 ng/ml LPS, and -more specifically- in individuals without progression of CWP only. The latter observation indicates that during the course of disease the ability of monocytes to release TNF upon stimulation may change (chapter 8 and chapter 10). In addition, compared to the original study in active miners, spontaneous release of TNF from monocytes was significantly reduced in retired miners compared to non-exposed controls (chapter 8). These observations suggest that baseline release of TNF is determined by active or current exposure, while stimulated release of TNF is related to past (cumulative) exposure, or to the presence of accumulated mineral dust in the lung. This is in contradiction with TGF data where both spontaneous and dust stimulated release (measured in the same monocyte supernatants) was higher in retired miners compared to the non-exposed individuals (chapter 10). The relation between monocyte release of TNF, TGF and IL-6 was described in chapter 10. Generally, IL-6 release from monocytes was found to be comparable to TNF release, except in silica incubations. This might be closely related to differences in the hazards of silica versus coal dust [Gosset et al., 1991], as well as differences in silicosis versus CWP pathology.

Most interestingly, stimulated release of TNF and TGF were positively correlated in non-exposed individuals, but inversely related to each other in subjects with CWP. TGF has recently been forwarded as an important regulator of GSH, [Arsalane et al., 1995], and decreased GSH status is considered as an important risk factor of fibrosis [Cantin et al., 1990]. In line with these observations, we found a negative correlation between monocyte TGF release and erythrocyte GSH (chapter 10). An important question still to be answered in

view of these observations is the concept of priming. Further research is needed to explain why different *in vitro* responses of peripheral blood monocytes reflect differences in the lungs of non-exposed, exposed, formerly exposed, and diseased subjects.

Gathering the evidence from *in vivo* animal studies and the epidemiological follow-up, the crucial role of TNF in mineral dust induced fibrosis is now established [Piguet et al., 1990], and the priming of monocytes in retired miners as well as increased TNF receptor levels suggest that systemic effects may occur in response to mineral dust exposure. Important to health surveillance, the risk for progression of CWP is related to individual monocyte TNF release. Whether or not these phenotype variations in TNF release are related to a genetic predisposition, e.g. based on polymorphisms of TNF promotor regions [Pociot et al., 1993] needs further study. TGF appears another potent candidate crucially involved in (the progression of) CWP (to PMF) [Vanhée et al., 1995]. Prospective follow-up of the present cohort, as well as external validation in other cohorts of miners will elucidate the predictive power of combined measurement of TNF and TGF and/or TNF and its receptors. These studies are currently in progress, using a simplified screening assay using whole blood incubations [Schins et al., 1996].

COLLAGEN

In chapter 12 we investigated whether serum PIIP levels can be considered as a biomarker of chronic coal dust exposure or of coal dust induced respiratory disorders, including fibrosis and obstructive lung disease. PIIP levels were neither related to lung function parameters (spirometry, impedance measurement, diffusion capacity), nor to chest radiography lesions as visualized by conventional chest radiography or high resolution computed tomography. Furthermore PIIP levels were not related to cumulative dust exposure or to any other parameter of exposure, although PIIP levels tended to decrease with increased cumulative exposure in miners with CWP (chapter 12). These negative findings are in sharp contrast to positive studies related to mineral dust exposure and/or lung function in subjects exposed to asbestos [Cavalleri et al., 1991], as well as in sarcoidosis patients [Bjermer et al., 1986]. Longitudinal analysis of PIIP levels in active coal workers in relation to radiological progression of CWP (described in chapter 11) did not forward PIIP as a susceptibility marker to development or progression of pneumoconiosis, opposite to observations in sarcoidosis patients [Pohl et al., 1992]. Unfortunately, we are not able to discriminate between retirement, normal intra-individual variation, or drift in the assay to explain for the significant differences in individual PIIP release at the two time points measured. However, the lack of "abnormal" high PIIP

values at follow-up (i.e. values above $2 \times \text{S.D.}$ of non-exposed individuals) is in favour of the first option, i.e. a reduction of serum PIIP in response to cessation of mineral dust exposure. However, a (positive) correlation between PIIP levels and cumulative exposure which would have supported this hypothesis, was not found at the two time points measured (chapters 11 and 12). All in common these data are not in favour of PIIP as useful biomarker in mineral dust exposure. Nevertheless, it may seem relevant to determine the predictive values of measurement of combined collagens such as PIIP in combination with type VI related collagen, since this is considered to allow for parallel assessment of both collagen synthesis and collagen degradation [Schuppan et al., 1994]. In addition, further studies (e.g. in situ analysis of collagens) are needed to determine the exact kinetics of type III collagen formation as well as other relevant collagens during the development of mineral dust induced fibrosis.

The use of “marker” approaches in occupational lung disease, and more specifically in relation to coal dust exposure is not new. For instance, nutritional status of miners has been considered as a risk factor explaining respiratory disorders in coal miners [Swartz et al., 1978]. In fact, lung function measurements themselves can be considered as important markers and have been used in routine screening of miners for many decades [Attfield and Wagner, 1982]. Interestingly, in many studies among coal miners a consistent relation between FEV1-decline and cumulative dust exposure has been reported in a range of 0.4 to $0.8 \text{ mL}\cdot\text{gh}^{-1}\cdot\text{m}^{-3}$ [Wouters et al., 1994]. Radiological stage of simple CWP, as well as its progression rate, have been regarded as indicative “markers” for the development of PMF [Gautrin et al., 1994]. Nowadays, HRCT analysis is applied to allow for a more sensitive detection of CWP, i.e. closer to the time of exposure in the exposure disease continuum (see chapter 2). Apart from differences in intrinsic biological responses, susceptibility differences in mineral dust induced disorders may also be related to internal or effective dose and determined by inter-individual variation in deposition of particles in the lung [Muir, 1991] or mucocilliary clearance [Bohning and Lippman, 1992] (see figure 13.1). In fact, observations in a sheep model of asbestosis, forwarded particle clearance as a susceptibility marker [Bégin et al., 1989; Borm, 1994]. Elucidation of the crucial role of elastases in emphysema and the large inter-individual variation of neutrophil elastases, has led to a denomination of this mechanism to explain for risk for lung emphysema [Karlinski et al., 1978; Hubbard et al., 1990].

A number of biological events or parameters occurring during coal dust exposure could be evaluated in this thesis (see figure 13.1), allowed validation of a set of biomarkers of mineral dust induced lung disorders, rejection of some

others, as well as generation of some new candidate markers. In Table 13.1 the most important findings are briefly summarized.

Table 13.1 Biomarkers evaluated in this thesis in relation to their application in molecular epidemiology of coal dust induced lung disorders

Marker	Event
TNF	progression of CWP, lung function decline #
TGF	chronic dust exposure
IL-6	presence of CWP
Vitamin A, E	obstructive lung disease
TRAP	progression of CWP (retrospective analysis)
SOD	progression of CWP, lung function decline
Catalase	progression of CWP, lung function decline
GSH/GSSG	Chronic bronchitis, progression of CWP
Gpx	Lung function decline, relation with GSH/GSSG, GST
GST	discrimination between interstitial and respiratory disease
8-oxodG/dG	Oxidative stress due to (former) exposure
PIIIP	Active fibrosis

Described in Jorna et al., 1994

It should be emphasized that the decision to recruit subjects involved in cross-sectional surveys for follow-up has allowed to test the validity of several of these biomarkers. The strength of this study (the prospective analysis) also brings along its weaknesses. Inherent to a longitudinal survey, markers initially measured, or the method by which they are analysed may be obsolete at the time of follow-up [Rothman et al., 1993]. Furthermore, the design of the original studies was case-control, and to investigate the involvement of cytokines, antioxidants and PIIIP in pneumoconiosis, miners were matched on age and exposure [Borm et al., 1990; Janssen et al., 1992a]. Statistical limitations are caused by the size of the population studied and the period of follow-up, in view of the incidence and prevalence of CWP. Indeed, more preferable methods to validate biomarkers could be applied, such as larger scaled prospective cohort studies (based on detailed power calculations), or multi-center studies [Schulte and Rothman, 1993]. Despite these limitations, the present approach allowed a fast validation of candidate biomarkers measured at earlier time. Furthermore, new candidates have been forwarded and related to the (validated) markers, at the same time leading to increased understanding of underlying biological mechanisms of coal dust induced lung disorders.

In relation to the implementation of present markers (see Table 13.1) the question rises, as to how far present markers can be extrapolated from "coal" workers to populations exposed to other mineral dusts (e.g silica or asbestos). Based on the common outcome of disease (chapter 1 and chapter 2) and the

similarity in their underlying biological mechanisms (chapter 3), at least for several markers this extrapolation may hold true: Considering the key role of the macrophage in mineral dust inhalation, as well as the suggested macrophage-fibroblast-pathway in its fibrogenic response [Heppleston and Styles, 1967], monocyte release of TNF most likely is a valid susceptibility marker in any mineral dust induced fibrogenicity. Evidently its exact predictive value needs to be assessed in other cohorts. Furthermore, the concomitant measurement of monocyte release of TGF and/or plasma soluble TNF receptors may have additional value, although they also need to be evaluated in a prospective survey. Serum and red cell antioxidants, particularly erythrocyte SOD activity and GSH concentration, have been validated by such a prospective design (chapter 7). Supported by several *in vitro* and *in vivo* observations (chapter 3), also the antioxidant route (see figure 13.1) most likely is a common mechanism in response to various mineral dusts and related disease, and indicate general application of red cell and serum antioxidant status as markers in fibrogenesis. Also in non-dust induced fibrogenic lung diseases, both cytokines (e.g. TNF, TGF) and ROS are considered to play important roles, and cytokine-antagonist (e.g. TNF antibodies) as well as antioxidants (e.g. oral N-acetylcysteine) are considered as promising therapies [Goldstein and Fine, 1995; Hunninghake and Kalica, 1995].

In sharp contrast, serum PIIP was rejected as marker of coal dust exposure, opposite to previous findings in relation to asbestos exposure [Cavalleri et al., 1988; 1991]. Furthermore, this marker was demonstrated to have predictive value in determining disease (progression) in sarcoidosis [Pohl et al., 1992], however not in CWP (chapter 11). Thus, unlike several cytokines and antioxidant factors, serum PIIP cannot be considered as a general marker of mineral dust exposure, or on the other hand of fibrotic lung disease.

The relations of some of the markers to nonpneumoconiotic effects, and their independence with pneumoconiotic effects in our studies, suggest a possible application beyond fibrogenic lung disease. An increase of TNF in obstructive lung disease in coal workers has been described previously [Jorna et al., 1994], and the relation between serum and red cell antioxidants and lung function decline, as well as between GSH and chronic bronchitis, was shown in this thesis. These findings may be confirmed, if the present cross-sectional and retrospective analyses of antioxidants and cytokines in relation to lung function decline and chronic bronchitis hold true in a prospective design i.e. by continuation of the follow-up of the present study group or in other surveys. Since lung function decline may be considered as a relatively non-specific response to a variety of mineral dusts (see chapters 1 and 2) as well as other exposures, the present markers are likely to have significant meaning in other occupational settings. To evaluate the use in the absence of occupational exposure, evidently further study in non-exposed individuals is needed. The relevance of oxidative DNA damage

in lymphocytes in relation to various lung disorders seems closely related to antioxidant parameters evaluated. This parameter may therefore be considered as valid marker of oxidative stress in response to chronic inhalation of mineral dusts. However, in relation to the cancer risk associated with (some) mineral dusts, the practical use of this marker is severely limited by the incidence and prevalence of these disorders, as well as their long latency.

The hallmarks of “molecular epidemiology” -although some molecular biologists and epidemiologists continue to debate on the rudeness of this formulation- are inherent to curiosity of mankind since the dawn of history: First, there is our need to explain why and, more important, how we are dealing with diseases. In the population studied in this thesis, the causal agent is known (see chapter 2). *In vitro* and *in vivo* studies (chapter 3) have lead to the clarification of important biological mechanisms playing a role in the development or progression of coal dust induced lung disorders. The studies described in this thesis further increased our understanding in these diseases.

Elucidation of the cause of a disease, almost directly leads to a second question: “why are we not all equally susceptible?” This question is obvious in the well known symbolism of two colliers who worked shoulder to shoulder for many decades at the same pit, the one in perfect shape, the other one suffering from severe shortness of breath. To explain (at least in part) for these aspects, in the studies described in the present thesis, several markers of mineral dust induced lung disorders were generated. The next step is to use these markers. This inevitably needs extrapolation of sophisticated, expensive and time consuming intra-laboratory methods, into simplified assays to implement these markers in large populations in workplace settings where laboratory conditions are limited. As stated before, these studies are currently in progress. The use of these markers has also ethical consequences [Schulte, 1991], since application of validated biomarkers, may “discriminate” individuals beyond the biological framework of (occupational) exposure and resultant disease. Both ethical and legal aspects of the use of biomarkers will need considerable attention in the future.

It is recommended to further evaluate presented markers -or combination of markers- in relation to specific research questions, such as their predictive value in coal workers at the start of their employment, in PMF patients, as well as in relation to other (mineral) dust exposures including workers employed in ceramic industry, in grinding or sandblasting, surface miners, construction workers etc. The work presented in this thesis may serve as a guide to implement both the presented framework of biomarkers (see figure 13.1) of mineral dust related disease and the generated (candidate) markers (see Table 13.1) in such surveys.

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Elk meetbaar biologisch effect tussen blootstelling en een daaruit resulterende ziekte kan men als "biomarker" bestempelen. Dit proefschrift beschrijft de evaluatie van een aantal biomarkers in relatie tot blootstelling aan mineraal stof. Aan de hand van een 5-jaar follow-up van mijnwerkers (Kempense Steenkoolmijnen, België) die oorspronkelijk bij diverse dwarsdoorsnede-studies betrokken zijn geweest, is onderzocht in hoeverre deze parameters in het bloed relevant zijn voor mensen die (beroepsmatig) zijn blootgesteld aan mineraal stof en derhalve mogelijk een verhoogd risico lopen op het ontstaan dan wel voortschrijden van aan deze blootstelling gerelateerde ziekten.

In **hoofdstuk 1** wordt een kort overzicht gegeven van de voornaamste soorten longaandoeningen die kunnen ontstaan bij blootstelling aan bekende en veel voorkomende vormen van mineraal stof zoals asbest, silica en mijnstof. Naast stof-specifieke ziektebeelden (bijvoorbeeld mesotheliom door asbest) veroorzaken diverse soorten mineraal stof ook min of meer gemeenschappelijke gezondheidseffecten waaronder longfunctie-verlies en longfibrose. Tot de voornaamste gezondheidseffecten die kunnen optreden na inhalatie van mijnstof (samengevat in **hoofdstuk 2**) behoren mijnwerkerpneumoconiose (MWP), chronische bronchitis, vermindering van longfunctie en emphyseem. De vraag of (silica bevattend) mijnstof al dan niet (long)kanker kan veroorzaken is vooralsnog niet opgehelderd en is momenteel (oktober '96) onderwerp van een IARC-werkgroep. MWP wordt gediagnostiseerd door beroepsanamnese en een thoraxfoto aan de hand van standaard criteria vastgesteld door het "International Labour Office" (ILO), waarbij MWP in diverse stadia geclassificeerd wordt op grond van aantal en grootte van opaciteiten. In tegenstelling tot progressieve massieve fibrose (PMF) is er bij milde vormen van MWP slechts sprake van geringe longfibrose. Het ontstaan van longfibrose uit zich in een excessieve toename in depositie van collagenen.

De biologische mechanismen die ten grondslag liggen aan de door mijnstof geïnduceerde aandoeningen worden beschreven in **hoofdstuk 3** aan de hand van *in vitro* en *in vivo* studies. Veelal wordt naast de effecten van mijnstof ook verwezen naar die van silica en asbest. Diverse soorten mineraal stof genereren aan de hand van directe (oppervlakte) eigenschappen van het stof dan wel via activatie van fagocyterende cellen (macrofagen, neutrofielen) reactieve zuurstofspecies (RZS). Wanneer de vorming van RZS dermate groot is dat antioxydatieve afweermechanismen waarover de mens beschikt niet afdoende zijn, kan oxydatieve schade optreden aan onder meer eiwitten, celmembranen (lipidperoxydatie) en DNA. Elke verandering in activiteiten of concentraties van antioxydanten kan worden beschouwd als indicatie voor deze zogenaamde "oxydatieve stress". Naast RZS produceren de door stof geactiveerde fagocyten (maar ook andere celtypen zoals alveolair epitheel) onder meer cytokines en groeifactoren die op hun beurt weer in staat zijn om secundaire cellen te

activeren en aan te zetten tot productie van diverse factoren (o.a. cytokines, RZS). Zo blijkt bijvoorbeeld dat mineraal stof macrofagen *in vitro* kan aanzetten tot de "release" van TNF. Dit cytokine blijkt in staat om *in vitro* de deling van fibroblasten te stimuleren en blijkt bij proefdieren het ontstaan van fibrose door silica aanzienlijk te versterken; anderzijds wordt in aanwezigheid van TNF-antilichamen in het zelfde model silicose grotendeels voorkomen. RZS en diverse cytokines worden in verband gebracht met het ontstaan van fibrose alsook andere longaandoeningen (emphyseem, kanker). Longfibrose ontstaat als gevolg van verhoogde depositie van met name door fibroblasten geproduceerde type III collagenen (in vroege lesions) en type I collagenen. De in dit proefschrift beschreven studies zijn opgesplitst in drie delen waarin achtereenvolgens markers van oxydatieve stress, cytokine release en collageen depositie zijn bestudeerd in relatie tot longaandoeningen bij mijnwerkers.

Het onderzoeksdesign (beschreven in **hoofdstuk 4**) is als volgt: 156 mijnwerkers (Kempense Steenkoolmijnen, België) participierend in drie studies uit 1987, zijn opgevolgd met het doel om de bij deze studies onderzochte parameters prospectief te relateren aan het ontstaan dan wel het voortschrijden van MWP. Dit is gebeurd op grond van gepaarde analyse van thoraxfoto's over een tijdsinterval van vijf jaar. Aan het eind van de follow-up zijn de betreffende markers opnieuw bepaald, en zijn tevens nieuwe markers bestudeerd (transversale en retrospectieve analyses). Om een mogelijke verband tussen de diverse parameters met blootstelling te bestuderen is de cumulatieve blootstelling aan respirabel mijnstof geschat aan de hand van "job-exposure" matrices, en is een controle groep van niet beroepsmatig aan (mineraal) stof blootgestelde personen meegenomen. Obstructieve longaandoeningen zijn door middel van spirometrie, impendatiemeting en vragenlijsten in kaart gebracht. In deelgroepen zijn additionele analyses uitgevoerd waaronder HRCT-scans en bepalingen van diffusiecapaciteit, en is retrospectief longfunctiedaling berekend over een periode van 10 jaar voorafgaand aan de oorspronkelijke studies.

Uit de resultaten van de follow-up (n=104) blijkt dat ondanks dat alle mijnwerkers niet meer actief zijn (vanwege algehele mijnsluiting in België) bij een aanzienlijk aantal personen progressie (45%) en in enkele gevallen zelfs nieuwvorming van MWP voor te komen (5%) (**hoofdstukken 8 en 11**). Schatting van de cumulatieve stofblootstelling toont een viervoudige variatie van blootstelling aan ten op zichte van het aantal jaren ondergronds (**hoofdstuk 12**), en blijkt gerelateerd aan de score van de thoraxfoto's, aan de HRCT-scans (**hoofdstuk 12**), én aan de progressie van MWP (**hoofdstuk 8**).

In **hoofdstukken 5 t/m 7** worden de oxydatieve DNA schade en antioxydatieve status besproken. Aan het eind van de follow-up is de antioxydatieve capaciteit van serum bepaald aan de hand van de TRAP-assay (**hoofdstuk 5**). Deze blijkt significant verhoogd te zijn bij mijnwerkers die gedurende de vijfjarige periode voorafgaand aan de bloedafname MWP ontwikkelden, zowel ten opzichte van

mijnwerkers zonder MWP als ten opzichte van mijnwerkers met reeds aanwezige MWP. Als maat voor oxydatieve DNA schade is in het DNA van lymfocyten de hoeveelheid 7-hydro-8-oxo-2'-deoxyguanosine bepaald (**hoofdstuk 6**). De schade blijkt niet te verschillen tussen mijnwerkers met of zonder MWP; deze is echter wel verhoogd bij mijnwerkers ten opzichte van niet-blootgestelde controles. De verhoogde oxydatieve schade in de lymfocyten van de mijnwerkers staat in mogelijk verband met de door anderen aangetoonde aanwezigheid van stabiele radicalen (en meer specifiek in de lymfeklieren) in longbiopten van (ex) mijnwerkers. In **hoofdstuk 7** zijn diverse antioxidanten gerelateerd aan prospectieve veranderingen in MWP, aan longfunctiedalingen (retrospectief), en aan chronische bronchitis. Tevens wordt in dit hoofdstuk de relevantie van de gecombineerde analyse van diverse antioxidant parameters in relatie tot pneumoconiose alsmede obstructieve longaandoeningen besproken. Voornaamste bevindingen zijn een verhoogde SOD-activiteit in erythrocyten van de personen die vervolgens MWP ontwikkelden, en een verlaagde GSH concentratie in chronische bronchitis. Aangezien zowel *in vitro* als *in vivo* is aangetoond dat verhoogde MnSOD expressie een indicator van oxydatieve stress is, zou men de verhoogde SOD-waarden in erythrocyten van personen met progressie van MWP kunnen verklaren als een weerspiegeling van verhoogde pathogene activiteit van stof in de long. Een verlaagde GSH activiteit in bronchoalveolaire lavage bij obstructieve longaandoeningen is door andere onderzoekers beschreven. In algemene zin tonen de in dit deel van het proefschrift beschreven resultaten aan dat oxydatieve stress, alsmede de hiermee geassocieerde oxydatieve DNA schade in personen blootgesteld aan mineraal stof in verhoogde mate aanwezig zijn buiten de long c.q. in perifeer bloed, én zelfs aanwezig blijven ondanks een (jarenlange) afwezigheid van deze (beroepsmatige) blootstelling.

De **hoofdstukken 8 t/m 10** behandelen achtereenvolgens de *ex vivo* release van TNF door perifere bloed monocyten (**h8**), de plasma concentraties van de 55kD en 75kD receptoren van TNF (**h9**), en de gecombineerde evaluatie van TNF, TGF β en IL-6 (**h10**). In overeenstemming met de rol van TNF in silicose bij de muis, indiceren de resultaten dat dit cytokine ook een cruciale factor is bij fibrose geïnduceerd door mijnstof. De herhaalde dwarsdoorsnede analyse toont aan dat release van TNF na *ex vivo* stimulatie van monocyten door mijnstof zowel in actieve als in ex-mijnwerkers verhoogd is ten opzichte van de niet blootgestelde controles, en laat tevens zien dat deze release in beide studies een afnemende trend vertoont met een toenemende gradatie van MWP. Voorts blijkt uit prospectieve analyse dat zowel een verhoogde TNF release, alsook de cumulatieve stofblootstelling de progressie van MWP verklaren. Stofblootstelling en TNF release zijn echter niet aan elkaar gecorreleerd. De gestimuleerde TNF release op beide meetpunten is duidelijk gecorreleerd, echter met name in de mijnwerkers zonder progressie, hetgeen indirect wijst op een invloed van het

ziekteproces op de release van TNF door monocyten. De relatief stabiele fenotypische variaties in TNF release berusten mogelijk op in het kader van andere aandoeningen beschreven polymorfismen van het TNF-promotor gen. In tegenstelling tot de gestimuleerde TNF release blijkt de spontane TNF release verlaagd te zijn in de ex-mijnwerkers ten opzichte van de actieve mijnwerkers. Waarschijnlijk wordt deze spontane release met name door "actuele" blootstelling bepaald, terwijl de gestimuleerde release gekoppeld is aan blootstellingsverleden, al dan niet bepaald door een voortdurende reactie in de long op geaccumuleerd mijnstof. Daarentegen blijkt de release van TGF β zowel spontaan als na *ex vivo* stimulatie verhoogd te zijn in de ex-mijnwerkers, en in beide gevallen verhoogd ten opzichte van de nooit blootgestelden. IL-6 release vertoont overeenkomsten met die van TNF, met uitzondering van de door silica gestimuleerde IL-6 release die nagenoeg gelijk blijkt te zijn aan de baseline release van IL-6. De *ex vivo* gestimuleerde release van TGF β en TNF is positief gecorreleerd in niet blootgestelde controles, maar negatief in de mijnwerkers met MWP, hetgeen wijst op de mogelijk tegengestelde rol van beide cytokines in de pathogenese van MWP. Tevens zijn bij de mijnwerkers ook verhoogde plasma concentraties van de TNF receptoren (sTNF-R55 en -R75) gevonden ten opzichte van de niet blootgestelde controles, en blijkt dat medicijngebruik ten tijde van bloedafname een verhogend effect op sTNF-R55 concentraties heeft. Prospectief onderzoek is echter nodig om te bepalen in hoeverre de sTNF-R's, TGF β en IL-6 in navolging van TNF een voorspellende waarde hebben met betrekking tot de progressie van MWP.

In de hoofdstukken 11 en 12 is de rol van serum type III procollageen peptide (PIIIP) bestudeerd. In tegenstelling tot het oorspronkelijke dwarsdoorsnede onderzoek is bij de ex-mijnwerkers geen verband tussen PIIIP en thorax of HRCT score aanwezig, en blijkt PIIIP niet gerelateerd aan de longfunctie (spirometrie, impedantiemeting, diffusiecapaciteit) (hoofdstuk 12). Bovendien blijkt er geen verband met enige parameter van blootstelling te zijn, uitgezonderd een negatieve correlatie tussen cumulatieve stofblootstelling en PIIIP in personen met MWP. De resultaten staan in contrast met andere studies waarbij PIIIP gerelateerd bleek te zijn aan asbestblootstelling en aan de ernst van sarcoidose. Longitudinale analyse toont aan dat serum PIIIP meting in MWP geen marker is voor de individuele gevoeligheid voor het ontstaan of de progressie van fibrose (hoofdstuk 11). Nadere studie is noodzakelijk om het precieze verband tussen serum PIIIP en processen van collageen depositie en afbraak zowel in de "gezonde" long als in MWP beter te begrijpen.

In dit proefschrift zijn diverse markers van blootstelling aan- en effecten van mijnstof bestudeerd en zijn parallel nieuwe inzichten in het ontstaan van longaandoeningen door blootstelling aan mijnstof gegenereerd. De vraag rijst nu in hoeverre de betreffende bevindingen geëxtrapoleerd kunnen worden naar

effecten van blootstelling aan andere soorten mineraal stof. Op grond van de resultaten beschreven in dit proefschrift en aanwijzingen uit dierexperimenteel en *in vitro* onderzoek lijkt dit "generaliseren" voor markers als TNF en antioxidant parameters meer op te gaan dan bijvoorbeeld voor PIIP. Enerzijds dus verdient het aanbeveling om de gepresenteerde bevindingen verder te evalueren voor mijnstof in relatie tot specifieke onderzoeksvragen zoals de voorspellende waarde van de markers in nieuwe mijnwerkers of in patiënten met PMF. Anderzijds echter kan ook aan onderzoek worden gedacht in het kader van andere vormen van blootstelling of andere soorten mineraal stof, zoals werknemers in de keramische industrie, of bij blootstelling aan stof tijdens processen als (steen)slijpen en zandstralen, en bijvoorbeeld in de bouw. De in dit proefschrift gepresenteerde studies kunnen hierbij als een soort leidraad dienen waarbij zowel het gepresenteerde raamwerk van biomarkers van longaandoeningen door mineraal stof alsmede de specifiek geëvalueerde markers in te passen zijn.

Voor velen zal na de titel en mijn naam, dit dankwoord waarschijnlijk zo'n beetje het eerste zijn dat gelezen wordt. Dat is maar goed ook, want men moet weten dat dit boekje weliswaar op mijn naam staat, maar dat vele anderen er zeker op de een of andere manier aan hebben bijgedragen. In de nu volgende dankbetuigingen zou ik "in het vuur van het spel" een enkeling kunnen vergeten. Daarvoor mijn welgemeende excuses.

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List of publications

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- Schins RPF, Gosset Ph, Wallaert B, Borm PJA. Multiple cytokines as biomarkers in coal dust exposure and pneumoconiosis: Tumor Necrosis Factor- α , Interleukin-6, and Transforming Growth Factor- β , submitted.
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