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MECHANISTICALLY IDENTIFIED SUITABLE BIOMARKERS OF EXPOSURE, EFFECT, AND SUSCEPTIBILITY FOR SILICOSIS AND COAL-WORKER'S PNEUMOCONIOSIS: A COMPREHENSIVE REVIEW

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Clinical detection of silicosis is currently dependent on radiological and lung function abnormalities, both late manifestations of disease. Markers of prediction and early detection of pneumoconiosis are imperative for the implementation of timely intervention strategies. Understanding the underlying mechanisms of the etiology of coal workers pneumoconiosis (CWP) and silicosis was essential in proposing numerous biomarkers that have been evaluated to assess effects following exposure to crystalline silica and/or coal mine dust. Human validation studies have substantiated some of these proposed biomarkers and argued in favor of their use as biomarkers for crystalline silica- and CWP-induced pneumoconiosis. A number of "ideal" biological markers of effect were identified, namely, Clara cell protein-16 (CC16) (serum), tumor necrosis factor-α (TNF-α) (monocyte release), interleukin-8 (IL-8) (monocyte release), reactive oxygen species (ROS) measurement by chemiluminescence (neutrophil release), 8-isoprostanes (serum), total antioxidant levels measured by total equivalent antioxidant capacity (TEAC), glutathione, glutathione peroxidase activity, glutathione S-transferase activity, and platelet-derived growth factor (PDGF) (serum). TNF-α polymorphism (blood cellular DNA) was identified as a biomarker of susceptibility. Further studies are planned to test the validity and feasibility of these biomarkers to detect either high exposure to crystalline silica and early silicosis or susceptibility to silicosis in gold miners in South Africa.

Considerable attention has recently been paid to the utilization of biomarkers in the prevention of occupational diseases, in the scientific literature. Different categories of biomarkers are used to (1) assess exposure, (2) identify early changes or effects of this exposure, (3) identify the initiation of pathological changes prior to development of disease state, and (4) predict underlying susceptibility of persons to disease. Biomarkers thus have great potential to improve the process of risk assessment in the working environment in general (Schulte, 1995) and in lung disease in particular (Borm, 1994; Kim et al., 2000).

Silicosis is currently used as the health outcome for crystalline silica dust dose-response assessments. Clinical detection of silicosis is dependent on the detection of radiological abnormalities, which are a late (10 to 15 yr following exposure) and irreversible manifestation of disease. Elucidation of the mechanisms of action of crystalline silica-induced fibrosis has contributed greatly to the identification of a number of biological responses that are involved in the pathogenesis of silicosis. As silicosis is not a curable disease, it is of great interest and practical consequence to investigate the

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possibility of using these biological responses as prospective markers, which may indicate early exposure to crystalline silica or early onset of silicosis. In addition, it is also of importance to assess these biological responses before the threshold burden of silica in the lung has been exceeded. It has recently been shown that once this threshold has been exceeded, silica-induced pulmonary disease progresses without further exposure to silica (Porter et al., 2004).

If scientifically acceptable potential biomarkers for crystalline silica dust exposure can be identified, they can be utilized for the early detection of unacceptably high levels of dust, rather than irreversible radiological changes in the lungs. As such, they may contribute significantly to the evaluation of dust-allaying measures in different industries and/or in the identification of subjects that may be susceptible to developing silicosis. To this end, a comprehensive literature survey was conducted to identify potential biomarkers for crystalline silica dust exposure, which were then evaluated and prioritized for further investigations. In this review, literature on coal-workers' pneumoconiosis (CWP) was included, in addition to that on silicosis, as coal dust may contain crystalline silica. There are also similarities in the mechanisms involved in crystalline silica- and coal dust-induced pneumoconiosis (Castranova & Vallyathan, 2000; Schins & Borm, 1999).

BIOMARKERS OF EXPOSURE

Mechanistic Justification Biomarkers of exposure give an indication of the presence of the toxic substance or its metabolites in the body and therefore can be used as measures of their internal doses (Schulte, 1995; Ward & Henderson, 1996). True to this definition, investigations reported in the literature have assessed exposure to crystalline silica either by direct measurement of the number of crystalline silica particles per se or by indirect measurements using chemical or microscopic techniques to measure the level of SiO_2 and silicon (Si) in cells isolated from bronchoalveolar lavage (BAL), from lung tissue, or in urine and blood samples.

Animal and Human Studies for Validation of the Biomarkers of Exposure to Crystalline Silica A number of investigators have used the chemical molybdenum-blue methodology (Absher et al., 1992; Adamson et al., 1994; Hemenway et al., 1990) to assess crystalline silica in animal lung tissues. Others have used electron microscopy (EM) on these tissue samples, as these particles could not be identified with light microscopy. For example, the presence of crystalline silica in biopsy and autopsy samples of lung tissues could be determined using EM with an electronic probe microanalyzer (Pariente et al., 1970), electron sound analysis (Pariente et al., 1972), and scanning electron microscopy (SEM) together with energy-dispersive x-ray microanalysis (EDXA) (Funahashi et al., 1975, 1977, 1984; Liebetrau et al., 1987; Morgenroth, 1979), or SEM with x-ray energy spectrometry (Lapenas et al., 1982; McDonald & Roggli, 1995; Pierini, 1982; Pintar et al., 1976). More recently, transmission electron microscopy (TEM) and energy-dispersive spectroscopy (EDS) and x-ray microscopy diffraction (XRD) have been recommended to quantify quartz particles in paraffin-embedded lung samples. With this combined methodology, a statistically significant linear relationship between quartz in various lung compartments and silicosis severity was observed in gold miners, but no significant linear relationship could be established between the duration of dust exposure and the lung burden (Dufresne et al., 1998). In another study, using SEM of silicotic lungs to evaluate the degree of airway thickening (fibrosis) and EDS to analyze the silicon content of the thickened areas, a correlation was found between the latter and the septal thickening of the lung (Siegesmund et al., 1985). In addition, using SEM and EDXA, high content of Si in the lung may be seen as of diagnostic value for silicosis because it could differentiate between pleura of silicotic subjects and those with no occupational exposures to crystalline silica (Ferrer et al., 1994).

The use of BAL to assess the content of crystalline silica and other nonfibrous inorganic particles has also been suggested for use in populations exposed in dusty workplaces (de Vuyst et al., 1987; Dumortier et al., 1989; Gaudichet et al., 1987). For example, crystalline silica particles were identified in BAL using EM with microanalysis (Johnson et al., 1986). The use of semi-quantitative x-ray microanalysis (SXM) was also suggested as a less time-consuming procedure than EM microanalysis. Using SXM, it was possible to discriminate between silicosis and fibrosis

resulting from exposure to inorganic dusts other than crystalline silica (Funahashi et al., 1984; Lusuardi et al., 1992; Monso et al., 1997). A combination of SEM and SXM to assess crystalline silica content on BAL and sputum samples was also recommended (Nugent et al., 1989; Perna et al., 2002). Using this same methodology, however, the presence of crystalline silica in alveolar macrophages in BAL could not be confirmed although other mineral particles could be identified (Johnson et al., 1986). Nevertheless, there was a positive correlation between the silicon to sulfur content (Si/S) ratio and exposure to crystalline silica and silicosis; there was no correlation with the duration of exposure to crystalline silica (Lusuardi et al., 1992). With EM microanalysis, a correlation was also seen between the relative counts of inorganic particles in BAL fluid and the crystalline silica content of lung tissue, and thus it could be suggested that EM microanalysis of BAL fluid be used to assess exposure to crystalline silica (Chariot et al., 1992). Other biological fluids such as blood and urine were also used to assess exposure to crystalline silica by measuring the concentration of silicon in patients at different stages of silicosis (Rozenberg, 1966).

Comments on the Suitability of the Biomarker Although the presence of crystalline silica particles could be used to indicate exposure, neither its presence nor its elemental analysis in BAL, serum, or urine produced an accurate assessment of its internal dose in relation to disease. In addition, methodologies employed usually required expensive and highly sophisticated instrumentation. Therefore, the use of these biomarkers of exposure cannot be recommended for routine laboratory measurements, nor can they be used to establish accurate dose-response relationships in the production of disease. They may, however, be useful in experimental and pathological studies to elucidate tissue localization of crystalline silica particles and to provide some indication of local and total lung dose.

BIOMARKERS OF EFFECT

Biomarkers of effect either indicate early processes preceding disease or predict the development and presence of disease (late effect) (Bennett & Waters, 2000; Schulte, 1991; Ward & Henderson, 1996). These biomarkers may be the products of different cellular responses following exposure to the toxic substance, leading to the production of a variety of biomolecules. This review has shown that the great majority of investigations in crystalline silica-induced pathology concentrated on the identification of biological responses as predicted outcomes of the elucidation of mechanisms of action of crystalline silica toxicity. These responses could then be classified as early or late biomarkers of effect, depending on their ability to measure either the extent of initial interaction of crystalline silica with the cellular systems, or early minimum damage to the lung. Such early and late pulmonary responses following exposure to crystalline silica, assessed by characteristic early and late biomarkers, have been demonstrated in experimental animals (DiMatteo et al., 1996). An ideal biomarker of effect would be one that could measure early reversible events.

Early Response

Biomarkers of effect that would identify early events following exposure to crystalline silica were investigated. These biomarkers of early changes associated with functional response could then be correlated to exposure to crystalline silica per se rather than to the presence of silicosis.

Oxidative Damage Markers

Mechanistic justification Free radicals, generated either by the surface activity of crystalline silica or by the inflammatory response invoked by crystalline silica and the ensuing oxidative stress, feature prominently in the elucidation of processes preceding silicosis (Ghio et al., 1990; Porter et al., 2001; Shi et al., 1998; Vallyathan et al., 1998). Clinical work on exposed human subjects has confirmed the presence of stable free radicals in the lung tissue of autopsied coal miners (Dalal et al., 1991). Other groups have also shown increased radical generation by BAL cells from coal

miners (Kuempel et al., 2003; Wallaert et al., 1990). This confirmed previous animal studies that showed, for example, that coal-dust exposure upregulates the capacity of the alveolar macrophages (AM) to produce ROS, such as H_2O_2 and $O_2^{\cdot -}$ anion radicals (Castranova et al., 1996). As a result, several redox-sensitive transcription factors and receptors (e.g., NF-κB, EGF-R) (Hubbard et al., 2002) connecting oxidative stress to downstream events, such as production of cytokines and inflammation (Rom et al., 1987), lipid peroxidation and DNA damage (Bezrukavnikova et al., 1988; Daniel et al., 1993; Jajte et al., 1988; Knaapen et al., 2004; Shi et al., 1995), nitric oxide production (Blackford et al., 1997; Huffman et al., 1998), and cell proliferation and deposition of collagen (Benson et al., 1986; Friemann et al., 1999; Reiser et al., 1983), are listed as possible biomarkers that have been described in vivo in animals and in exposed human subjects. The justification for choosing these biomarkers was based on the following premises ensuing from a number of in vitro and in vivo animal experiments:

1. ***Free radical generation by surface activity of freshly fractured crystalline silica***. While investigating the role played by the surfaces of crystalline silica in its toxicity, it became apparent that free radicals are generated especially on the freshly fractured surfaces created during grinding (Figure 1). For example, when quartz was ground in a solution of hydrogen peroxide, two dinuclear oxygen species, $O_2^{\cdot -}$ and peroxy $SiOO^{\cdot}$ radicals, were formed at the surface and in the solution, through a catalytic decomposition of the hydrogen peroxide (Giamello et al., 1990). Studies prior to this showed that freshly fractured crystalline silica, when ground in air, had siloxyl radicals on the fracture planes, which could then generate hydroxyl radicals in aqueous medium (Shi et al., 1988; Vallyathan et al., 1988). Subsequently, it was shown that freshly

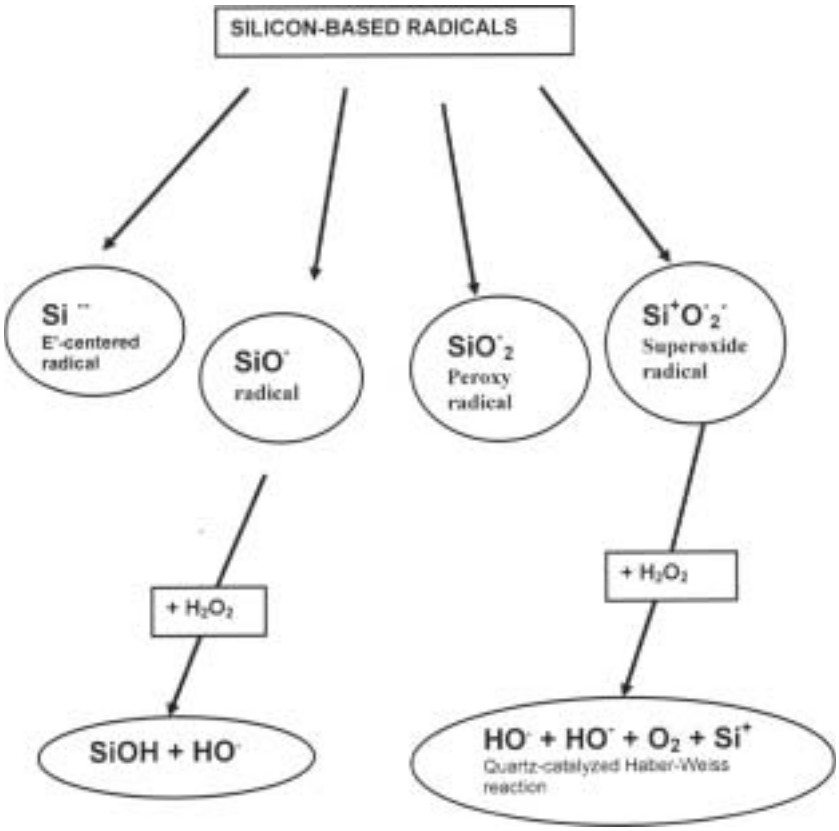

FIGURE 1. Schematic presentation of the free radicals and other reactive oxygen species (ROS) generated on the surface of crystalline silica.

ground quartz was more pathogenic than aged quartz in a rat inhalation model (Shoemaker et al., 1995).

2. ***Production of higher levels of hydroxyl radical by freshly fractured crystalline silica contaminated with iron***. When freshly fractured quartz was contaminated with iron, it produced higher levels of reactive oxygen species in water compared to the uncontaminated samples. When animals were exposed to these iron-contaminated samples, they produced higher levels of leukocyte recruitment, and the macrophages of the exposed animals produced higher levels of reactive oxygen than normal animals. It was therefore concluded that inhalation of freshly fractured quartz contaminated with trace levels of iron may be more pathogenic than inhalation of quartz alone (Castranova et al., 1997).
3. ***Role of released or adsorbed iron from body fluids on crystalline silica and coal mine dust to generate hydroxyl radicals and initiate lipid peroxidation***. The level of iron present in coal dust collected from different mining sites correlated well with the prevalence of pneumoconiosis at these sites and also with the ability of these dust samples to initiate lipid peroxidation, activate activator protein-1 (AP-1) and nuclear factors of activated T (NFAT) cells, and downregulate transferrin receptor gene expression in alveolar type II cells in culture (Hu et al., 2003; Huang et al., 1998, 1999, 2002; Zhang et al., 2002; Zhang & Huang, 2002). The degree of bioavailability of this iron was consequently considered as a critical parameter to predict the prevalence of CWP, following exposure in coal mine dust (Huang et al., 1998, 1999).

   Surface-complexed iron from in vitro and in vivo sources played a crucial role in the pathogenesis of crystalline silica particles due to the ability of the complexes formed to generate hydroxyl radicals (Ghio et al., 1992). For example, complexation of iron by humic-like substances was thought to play a role in CWP (Ghio & Quigley, 1994). In addition, it was shown that inflammation and lung injury following instillation of crystalline silica in rats could be associated with, among other factors, the elevation of in vivo surface adsorbed iron (Ghio et al., 1994, 1996). The complexed iron was shown to be bioavailable, which, in turn, could increase the degree of lipid peroxidation.
4. ***Crystalline silica and ROS radical-induced lipid peroxidation and DNA damage***. Lipid peroxidation was increased in linoleic acid, red blood cells, epithelial type II cells, and alveolar macrophages exposed to crystalline silica and coal dust in vitro (Chvapil et al., 1976; Dalal et al., 1990; Gabor & Anca, 1974; Gabor et al., 1975; Shi et al., 1994, 1998; Zhang & Huang, 2002), as well as in lung homogenates, subcellular fractions, lung slices, the lavage fluid, or whole lungs (Gupta & Kaw, 1982; Jajte et al., 1987, 1988; Vallyathan et al., 1995, 1997; Zhang et al., 1996; Zsoldos et al., 1983) of animals exposed to crystalline silica in vivo. In addition, instillation of crystalline silica into the lungs of rats increased serum and BAL fluid lipid peroxides with linear correlation (Guo et al., 1995; Petruska et al., 1990). Lipid peroxide concentrations were also increased in blood plasma of silicotic and CWP patients (Baimanova, 1999; Bezrukavnikova et al., 1988).

   Naked DNA incubated with crystalline silica resulted in DNA strand breaks (Shi et al., 1994). In vitro exposure to crystalline silica at concentrations between 20 and 100 $\mu g/m^3$ produced DNA damage in alveolar macrophages (Zhang et al., 1999, 2000) and lung epithelial cells (Schins et al., 2002) detected by Comet assay and by the levels of 8-hydroxydeoxyguanosine (8-OHdG), respectively. However, considerable differences were noted in the genotoxic potential of crystalline quartz samples (>98% pure) in lung epithelial cells in culture. The strand breaks assessed by Comet assay were only increased with the concomitant increase of cytotoxicity to the cells (Cakmak et al., 2004). Damage of DNA in lung cells detected by these two methodologies was also observed in experimental animals following in vivo exposure to crystalline but not amorphous silica (Johnston et al., 2000; Knaapen et al., 2002; Rehn et al., 2003; Seiler et al., 2001; Yamano et al., 1995). Finally, dust-exposed and silicotic patients had higher leukocyte and urinary 8-OHdG levels, measured by high-performance liquid chromatography (HPLC), compared to controls (Yamano et al., 1995). Pottery and foundry workers had greater lymphocyte DNA damage, measured by Comet assay (Basaran et al., 2003), and coal workers had higher

blood lymphocyte 8-OHdG levels measured by HPLC (Schins et al., 1995b), compared to control subjects.

5. ***Crystalline silica-stimulated ROS and RNS production by alveolar macrophages***. The activation of "respiratory burst" in phagocytic cells, such as macrophages and neutrophils, by crystalline silica is considered one of the main mechanisms in the pathogenesis of silicosis. The oxidants generated through this process include the superoxide anion radical ($^{\cdot}O_2^-$) and hydrogen peroxide (H_2O_2), the latter of which can form hypochlorous acid (HOCl) in the presence of the enzyme myeloperoxidase (Castranova et al., 1996). A lucigenin-dependent chemiluminescence test was used to determine crystalline silica-induced superoxide anion radical and hydrogen peroxide levels (Zhang et al., 2000), and a luminol-dependent chemiluminescence test was used to determine silica-induced reactive nitrogen species (RNS) levels in rat alveolar macrophages (Blackford et al., 1997; Huffman et al., 1998; Porter et al., 2002a).
6. ***Activation of the transcription factor NF-κB and induction of gene expression for cytokines including TNF-α and growth factors by crystalline silica-induced ROS lead to inflammation, cell damage, fibrosis, and cancer***. The nuclear transcription factor NF-κB is one of the key oxidative stress-induced regulatory proteins (Schreck et al., 1992). This transcription factor regulates many effector genes, including those encoding cytokines and adhesion molecules (Wang et al., 2002). Its involvement in oxidant-mediated lung injury in a number of respiratory disorders has recently been reviewed (Maselli et al., 2002; Wang et al., 2002), as has its role in particle-induced lung disease (Schins & Donaldson, 2000). Its activation by crystalline silica has been demonstrated (Castranova et al., 2002; Chen & Shi, 2002; Hubbard et al., 2002; Kang et al., 2000a, 2000b), as has the depletion of its inhibitor I-κB (Schins et al., 2000) The association of NF-κB activation with crystalline silica-induced pulmonary inflammation and its inhibition with suppression of crystalline silica-induced inflammation have also been demonstrated (Sacks et al., 1998). Crystalline silica-induced TNF-α gene expression was proposed to be via crystalline silica-induced NF-κB (Driscoll, 2000; Hubbard et al., 2002; Rojanasakul et al., 1999; Shi et al., 2001), and both processes were suggested to be initiated via crystalline silica-mediated free radical generation (Chen et al., 1998; Gossart et al., 1996), leading to cellular damage and fibrosis (Porter et al., 2002b). This proposed mechanism of NF-κB activation induced by crystalline silica was therefore seen as a possible target for development of anti-inflammatory and antifibrotic drugs (Kang et al., 2000b).
7. ***Initial depletion and subsequent elevation of antioxidant levels in different cellular and biological fluid compartments as a compensation in response to crystalline silica-induced oxidative stress***. Antioxidant enzyme activities, such as superoxide dismutase (MnSOD, CuZnSOD) and glutathione peroxidase (GPx), are increased in the lungs of crystalline silica-exposed animals (Janssen et al., 1992b; Vallyathan et al., 1995, 1997, 2000). Increased levels of MnSOD in lung tissues have also been found by other investigators at early stages of silicosis in rats exposed to quartz (Ghio et al., 1994; Zsoldos et al., 1983), although the activities of this enzyme were decreased in BAL fluid (Yamano et al., 1995). Clinical studies have demonstrated an increase of MnSOD (but not total SOD) in patients with early and late stages of coal-worker's pneumoconiosis (Wallaert et al., 1990).

   Glutathione (GSH) content in silicotic lung tissues, on the other hand, was decreased in the early stages of disease (Ghio et al., 1994) and increased in the late stages (Yamano et al., 1995). GSH levels in red blood cells were also found to be decreased in early and increased in late stages of silicosis (Borm et al., 1986), while the level of this antioxidant was decreased in alveolar macrophages following their exposure to crystalline silica in vitro (Zhang et al., 1999).

Human studies for validation of the identified biomarkers of oxidative stress A substantial number of oxidants, antioxidants, and antioxidant enzymes were tested in red blood cells, serum, BAL fluid, and BAL cells to assess the degree of oxidative stress following exposure to crystalline silica, or in silicotic patients to validate the aforementioned premises resulting from in vitro and in vivo animal investigations. Since the antioxidant system is composed of many mutually dependent

components, integrative approaches were also implemented, using alternative assays, namely, (1) total radical-trapping antioxidant parameter (TRAP), (2) Trolox equivalent antioxidant parameter (TEAC), (3) Comet assay, (4) levels of 8-oxodG, malondialdehyde, or isoprostane, or (5) alternative analyses (sums of antioxidants), as predictors of effect.

One of the biological samples used for human validation studies was BAL fluid. Alveolar macrophages isolated from BAL fluid of crystalline silica-exposed individuals with concomitant respiratory impairment were found to release significant amounts of superoxide anion radical and hydrogen peroxide compared to cells from nonexposed individuals (Rom, 1991; Wallaert et al., 1990). The production of ROS by alveolar inflammatory cells isolated from BAL of patients with progressive massive fibrosis (PMF) was higher than that from patients with simple pneumoconiosis (Wallaert et al., 1990). The activities of different antioxidant enzymes, such as GPx and SOD, as well as lipid peroxidation measured as isoprostane levels, were also found to be increased in the acellular fraction of BAL fluid of coal-dust-exposed miners with CWP compared to those without CWP (Vallyathan et al., 2000). Results with SOD activity in BAL fluid were later confirmed in a similar study, and it was found that the level of enzyme activity rose linearly with the lung burden of quartz (Kuempel et al., 2003).

The most important validation studies for biomarkers of oxidative stress, however, used peripheral blood components. Early work on 19 silicotic patients and 48 control subjects indicated an increase in GSH levels with no significant increases in activity of GPx and SOD or in lipid peroxidation in red blood cell lysate of CWP patients compared to control subjects (Borm et al., 1986, 1987). In contrast, the levels of GSH were decreased in conjunction with glutathione *S*-transferases in the red blood cells of coal miners in the early stages of pneumoconiosis (Evelo et al., 1993). The decrease in GSH levels in red blood cells in early stages of pneumoconiosis was in agreement with another investigation, which, however, found higher levels of antioxidant enzyme GPx activity with no changes in SOD activity (Engelen et al., 1990).

SOD activity in red blood cell lysates was found to be increased in underground miners compared to a group of surface miners, and it was concluded that erythrocyte Cu^{2+}/Zn^{2+} SOD activity may be a marker of effect of respirable coal-mine dust in exposed workers. In this same study, the GPx activity levels were also found to be higher in the plasma of retired miners with pneumoconiosis compared with those without pneumoconiosis. No such difference in serum total antioxidant content was seen between these two groups (Perrin-Nadif et al., 1996). Similarly, when total serum antioxidant content was measured by the TRAP assay, no significant differences were seen between non-dust-exposed controls, exposed miners without CWP, and those with CWP. However, TRAP was significantly increased in new cases of CWP, and there was an inverse relationship between GSH content and the stage of CWP. These results led to the conclusion that during the early stages of pneumoconiosis oxidative stress is present in the lung, reflected by the changes in concentration of these antioxidants and antioxidant enzymes in the peripheral blood, reflecting exposure to dust or different stages of CWP (Schins et al., 1994).

Later studies could not confirm these reported differences (Perrin-Nadif et al., 1998). However, the same group, in a more detailed study, did show a negative correlation between cumulative dust exposure and the erythrocyte SOD activity, and a positive correlation with erythrocyte catalase activity only in miners exposed to high dust concentrations with recent exposures. They also reported a correlation between catalase activity in relation to the severity of CWP, confirming the premise that production of ROS may be an important event in exposure to coal mine dusts and the severity of CWP (Nadif et al., 1998).

Plasma selenium (Se) concentration, as an antioxidant trace metal ion, was also determined. Plasma of workers exposed to coal dust over a long period with current exposure had lower concentrations of Se than those with long-term exposure and no current exposure to coal dust (Oryszczyn et al., 1996). The low concentration of plasma Se was later confirmed and found to be correlated with low plasma GPx activity in active miners with high dust exposures. It was concluded that variation in Se concentration in relation to changes in occupational exposure to coal dust reflected its protective role against reactive oxygen species generated by exposure to coal mine dust (Nadif et al., 2001).

Apart from the generation of ROS or responses by anti-oxidant levels, related pathways investigated included oxidative damage to DNA. Two in vivo studies were reported on oxidative DNA damage in peripheral blood lymphocytes of quartz-exposed workers. Data suggested that particle exposure per se produced an increase in oxidative DNA damage, irrespective of the induction or presence of CWP by inhaled particles (Schins et al., 1995b, 2002). No such correlation was seen in quartz-exposed workers (Pilger et al., 2000).

Comments on the suitability of the biomarkers of oxidative stress There is sufficient evidence in the literature, with substantial in vitro and in vivo animal investigations followed by validation studies with human subjects, to conclude that oxidative stress is a major process involved in silicosis. The validation of some of these oxidative stress parameters included (1) generation of ROS from AM, (2) activation of NF-kB, (3) total radical trapping antioxidant capacity, (4) isoprostanes and GSH levels in serum, (5) GPx and SOD activites in erythrocytes, and (6) DNA damage in lymphocytes. These parameters have the potential to be reliable biomarkers for early effects of exposure to crystalline silica.

It may be problematic to assess the production of ROS by human alveolar macrophages due to the logistics involved in its availability, but this can be substituted by assessing the production of ROS by peripheral polymorphonuclear leukocytes isolated from exposed subjects following their ex vivo exposure to crystalline silica (Hedenborg & Klockars, 1989; Maly, 1988). Another difficulty is that the same biomarkers of oxidative stress may be changed by mineral particles other than crystalline silica (Gulumian, 1999; Holley et al., 1992; Janssen et al., 1992b) and therefore may lack specificity. However, this may be overcome by the fact that the level, if not the type, of some of these parameters in peripheral blood was found to be a differentiating factor in different types of mineral particles (Kamal et al., 1989).

Neopterin as a Biomarker for Crystalline Silica Exposure

Mechanistic justification Neopterin, 6-d-erythro-trihydroxypropyl-pteridin, and its reduced dihydroform, 7,8-dihydro-neopterin, are produced by monocytes/macrophages upon stimulation with interferon-γ (Murr et al., 1994, 1996). There is controversy in the literature about the role played by neopterins as antioxidants or pro-oxidants (Gieseg et al., 2001; Widner et al., 2000; Wirleitner et al., 2001), but it seems that pH, the presence of chelated iron, and the type of reactive oxygen species may determine their pro-oxidant or antioxidant characteristics (Oettl et al., 1999; Wede et al., 1999; Weiss et al., 1993). The production of neopterin by monocytes/macrophages has been reported to be closely related to the capacity of the cells to release toxic metabolites, especially ROS, and therefore an increase in its concentration in body fluids could be an indirect estimate of cell-mediated oxidative stress (Murr et al., 1999). Neopterin was also shown to induce apoptosis in T lymphocytes (Wirleitner et al., 2003) and in alveolar epithelial cells (Schobersberger et al., 1996).

Several studies have shown that in diseases related to immunity, rheumatoid arthritis, and malignancies, neopterin is elevated and may be a valuable biomarker in the clinical diagnosis of progression of disease (Altindag et al., 1998, 1999; Berdowska & Zwirska-Korczala, 2001; Bogner et al., 1988; Schobersberger et al., 1996; Wachter et al., 1989).

Human studies for validation of the identified biomarker of oxidative stress, neopterin Recently, in a study of 22 crystalline silica-exposed workers and 20 healthy volunteers, serum and urine neopterin levels were reported to be increased in an exposed population. It was proposed that neopterin was a new and useful early marker for the prediction of some disorders related to occupational crystalline silica exposure (Altindag et al., 2003).

Comments on the suitability of the biomarker From a single human validation study, it appears that the determination of neopterin levels in plasma may be a useful early biomarker following exposure to crystalline silica if combined with other biomarkers. However, further validation studies are necessary in experimental animal models as well as in case-control studies in humans to confirm this observation.

Clara Cell Protein-16 (CC16) Levels in Serum

Mechanistic justification Clara cell protein-16 is a homodimer consisting of 70 amino acid subunits, and has a molecular mass of 15, 840 kD, hence the CC16 abbreviation (Bernard et al.,

1993). CC16 protein is secreted almost exclusively by the nonciliated (Clara) cells of the tracheobronchial epithelium, with very weak synthesis by some reproductive system organs, such as the prostate (Peri et al., 1993; Singh et al., 1988). The exact physiological function of CC16 remains unknown, but there are several lines of evidence indicating that it is an immunosuppressive and anti-inflammatory protein protecting the airways from undue activation of the immune system that might produce tissue injury (Doyle et al., 1998). Since CC16 secreted in the respiratory tract could diffuse passively across the bronchoalveolar–blood barrier into the plasma (Hermans et al., 2001), it was suggested that the concentration of this protein in plasma could be a measure of integrity of Clara cells and serve as a specific biomarker of lung injury (Bernard et al., 1998). Decreased concentration of CC16 has also been associated with increased recruitment of fibroblasts in fibrosing lung disorders (Lesur et al., 1995).

Human studies for validation of the identified biomarkers of early response of cell injury It has recently been shown that the concentration of CC16 in serum from workers exposed to crystalline silica averaged 12.3 μg/L, versus 16.3 μg/L in controls, with no changes in respiratory symptoms and lung function tests (Bernard & Hermans, 1997; Bernard et al., 1994; Doyle et al., 1998). It was suggested that a significant reduction of serum CC16 in workers inhaling crystalline silica-rich dust could be used as a biomarker of early toxicity of exposure to crystalline silica and subsequently improve the detection of groups at risk. A plausible mechanism through which crystalline silica could alter serum concentration of CC16 was thought to be a reduced secretion of this protein into the respiratory tract due to crystalline silica-induced lung injury, leading to decreased concentration of CC16 in serum. An earlier animal study reported Clara-cell hyperplasia in rat lung following exposure to crystalline silica and coal mine dust (Albrecht et al., 2001). However, this study was not able to confirm or reject the aforementioned mechanism of reduced CC16 secretion in serum following exposure to crystalline silica, as it did not assess the levels of this protein concomitantly with Clara-cell hyperplasia.

Comments on the suitability of the biomarker There was a correlation between CC16 in serum and BAL fluid in humans (Bernard et al., 1992; Broeckaert et al., 2000; Shijubo et al., 1997). This low-molecular-weight protein would therefore make a reliable biomarker of early effects of crystalline silica toxicity, especially if its decrease is associated with exposure to crystalline silica per se, rather than with crystalline silica-induced lung impairment.

Late Response

Release of a number of enzymes from different cellular compartments and different cell types was used as a biomarker of cytotoxic cell damage of the lung. The levels of these enzymes were assessed in alveolar and interstitial macrophages, lung cells, BAL fluid, and serum of crystalline silica-exposed animals and human subjects.

Lysosomal and cytosolic enzymes

Mechanistic justification Damage to the cellular membrane or to the different organelles in the cell by quartz could be a mechanism for the initiation of inflammation. Such cell damage will release enzymes specific to these cellular compartments into the extracellular space. Assessing the levels of these enzymes in the extracellular space therefore provides an indication of cellular damage. For example, increases in activities of the lysosomal enzymes β-*N*-acetylglucosaminidase and β-glucuronidase are used as indicators of lysosomal damage, and increases in the cytosolic enzyme lactate dehydrogenase (LDH) activity and the membrane-bound enzyme alkaline phosphatase activity are used as indicators of cell membrane damage by crystalline silica (Dethloff et al., 1986; Kim et al., 1999a; Wallace et al., 1985; Zhang et al., 1999).

Animal and human studies for validation of the identified biomarkers of late response of cell injury β-Glucuronidase activity was found to be increased in the lysosomal fraction of lung homogenates of crystalline silica-exposed animals (Jajte et al., 1988). Similarly, the level of this enzyme activity and levels of the lysosomal enzyme β-*N*-acetylglucosaminidase and the cytosolic enzymes lactate dehydrogenase (LDH) and alkaline phosphatase were found to be increased in lung lavage fluid in rats following exposure to crystalline silica (DiMatteo et al., 1996; Driscoll et al., 1990b;

Johnston et al., 2000; Knaapen et al., 2002; Lindenschmidt et al., 1990; Vallyathan et al., 1995; Zhang et al., 1996) and in alveolar macrophages of guinea pigs following inflammation at later stages of exposure to crystalline silica. This inflammation continued to increase even after cessation of exposure, with concomitant increase in activity of these enzymes (Sjostrand & Rylander, 1984; 1987). Increases in the activities of LDH and alkaline phosphatase were also reported in BAL fluid of crystalline silica-exposed workers. This increase was related to the severity of the disease process (Larivee et al., 1990). Similarly, an increase in the ratio of alkaline phosphatase activity to lung lavage fluid albumin concentration was taken as a reflection of progression of fibrosis (Capelli et al., 1997b).

The activities of these enzymes in serum of crystalline silica- or coal-dust-exposed rats and workers were also assessed. Rats inhaling high concentrations of coal-mine dust showed increased LDH activity in BAL (Donaldson et al., 1990). Total serum LDH activity was also increased, and there were changes in its isozyme pattern towards a high LDH3 in coal workers, even after long-term cessation of exposure from coal dust (Cobben et al., 1997). Serum activity of two lysosomal enzymes, β-*N*-acetylglucosaminidase and β-glucuronidase, was also assessed in silicotic patients. The activity of the former (but not the latter) enzyme was higher in these patients than in crystalline silica-exposed subjects with no adverse clinical symptoms (Koskinen et al., 1983a, 1984a, 1984b).

Comments on the suitability of the biomarker The aforementioned enzymes appear to be natural biomarkers of late effects for silica-induced cytotoxicity. This will certainly be true if measured in BAL fluid. However, if they are assessed in serum, these enzymes may lack specificity and therefore activity levels will give general background knowledge on cell damage. They should be assessed concomitantly with other biomarkers, unless the isozyme specific to lung is measured in this biological fluid (Capelli et al., 1997a; Cobben et al., 1997).

Angiotensin Converting Enzyme (ACE)

Mechanistic justification ACE is a peptidyldipeptide hydrolase in the renin–angiotensin system (1) converts angiotensin-I into the potent vasopressor angiotensin-II and (2) inactivates the vasodilator bradykinin, which is the product of the kallikrein–kinin enzyme system. ACE is located mainly on the luminal surface of vascular endothelial cells (Beldent et al., 1995) but is also present in monocyte–macrophage cells (Eklund et al., 1987). In silicosis and other dust-related lung fibrosis, both the endothelial cells and macrophages were considered to be the source of increased serum ACE levels (Brown et al., 1983; Gronhagen-Riska, 1979; Gronhagen-Riska et al., 1978; Nordman et al., 1984; Thompson et al., 1991; Zhicheng et al., 1986). The increase in the activity of ACE in BAL fluid and serum was considered to be a marker of lung injury in a number of pulmonary diseases because its concentration in the latter biological fluid is only a very small fraction of the total body ACE activity (Beneteau-Burnat & Baudin, 1991; Henderson, 1984; Orfanos et al., 2000; Rohatgi, 1982; Studdy et al., 1983).

Animal and human studies for validation of the identified biomarkers of late response of lung injury In this review, a substantial number of studies were identified that assessed the activities of serum ACE in experimental animals. These studies showed an increase in serum ACE activities in silicotic animals but either recommended (Lin, 1990) or could not recommend (Brown et al., 1983) it as a useful biomarker to monitor lung damage from exposure to crystalline silica.

Human studies have also produced contradictory results. Increased serum ACE activities were reported in silicotic patients compared to control subjects, but this activity did not reflect the severity of the disease as determined by chest x-ray changes and respiratory function tests, nor did it give further information on the progression of the disease (Bucca et al., 1984; Gronhagen-Riska, 1979). Other studies confirmed an elevation in serum ACE activities in silicotic patients but, in contrast to the previous studies, found an association between the serum ACE activity and the roentgenographic severity of fibrosis (Nordman et al., 1984; Serbescu & Paunescu, 1992; Yano et al., 1987). The elevated level of serum ACE in silicotic patients reported in these previous studies, however, could not be confirmed by yet another investigation (Romano et al., 1985).

Studies were also conducted where the serum ACE activities were compared between a number of lung diseases, including silicosis. The activities of this enzyme in the serum of sarcoidosis or

patients with other lung diseases were compared to those of silicotic patients (Calabro et al., 1990; D'Andrea et al., 1979; Gronhagen-Riska et al., 1978; Inoue et al., 1987). It was found that the ACE activities were significantly raised in all patients compared to controls. It was concluded that elevated ACE activity in both these diseases would weaken the differential diagnostic importance of this enzyme determination in these disease states, although very high values may still indicate sarcoidosis. A similar conclusion was also drawn by Fernandez Jorge and Alonso Mallo (1994), who studied serum ACE activity in sarcoidosis, tuberculosis, and silicosis and showed significantly increased values with respect to the control group in all of these diseases. Results presented by another group (Szechinski et al., 1986) suggested that serum ACE level determinations may be used to assist in distinguishing between silicosis and silicotuberculosis, but not silicosis and sarcoidosis.

In the investigations reported, serum ACE activities were assessed using a fluorescent chromophore or radioactively labeled synthetic peptide substrates. A criticism was that, under certain circumstances, this type of methodology may not reflect the true level of ACE activity in the samples and thus may provide false high values when other serum peptidases are present, or false low values in the presence of pharmacologic enzyme inhibitors (lgic et al., 1972; Klauser et al., 1979; Saldeen et al., 1981). Subsequently, the serum ACE activity was measured with direct immunoassay with concomitant enzyme activity and the specific activity of serum ACE was calculated (Brice et al., 1995; Hiwada et al., 1987). Using this methodology, both investigations confirmed the increase in serum ACE activity in silicotic patients compared to control values.

Contradictory results were also reported following measurement of serum ACE activities in CWP. It was found that serum ACE activities in either miners without recent exposure to coal-mine dust or those with CWP were elevated compared to control subjects. It was concluded that underground coal mining, but not CWP, was associated with elevations in serum ACE activity and that, after removal from exposure to mixed coal-mine dusts, the levels of serum ACE activity normalized (Thompson et al., 1991). In contrast, another investigation of CWP showed elevated serum ACE activity in CWP compared to control subjects but no correlation was seen with progression of disease (Wallaert et al., 1985). Serum ACE activities were also measured in anthrasilicotic patients and were found to be elevated to the same level as in anthracosilicotuberculosis patients, suggesting that the levels of ACE activity could not be used to differentiate between these two disease states (Zhicheng et al., 1986).

Comments on the suitability of the biomarker Despite a large number of clinical studies on ACE in human exposed subjects, no definite conclusions can be drawn. Increased serum levels of ACE activity may or may not be the expression of an active progressive state of silicosis; ACE activity may be the expression of exposure to mixed dust in CWP or an expression of CWP; and ACE activity may or may not be a useful parameter to differentiate between silicosis and other diseases.

Inflammation and Fibrosis—Cytokines, Monokines, Lymphokines, and Growth Factors Inflammatory response to crystalline silica is manifested by an increased numbers of macrophages, neutrophils, and lymphocytes (Begin et al., 1987; Davis et al., 2001; Donaldson et al., 1992; Driscoll, 2000; Driscoll et al., 1995, 1997; Rom et al., 1987; Sjostrand et al., 1991). Since the original finding of Heppleston and Styles (1967) that crystalline silica-exposed alveolar macrophages (AM) produce factors that stimulate the production of collagen by fibroblasts, the field of growth factors and cytokines has developed exponentially, and several cytokines have now been advanced as crucial biomarkers in particle-induced fibrosis (Gauldie et al., 1993; Jordana et al., 1993; Kelly et al., 2003; Lugano et al., 1984). Mediators of importance that were investigated included, among others, cytokines such as tumor necrosis factor (TNF-α) and interleukin 1 (IL-1), chemokines such as IL-8, and growth factors such as fibroblast growth factor (FGF), transforming growth factor (TGF), and platelet-derived growth factor (PDGF) (Borm et al., 1990; Driscoll et al., 1993; Gosset et al., 1991; Guoping et al., 1997; Kelley, 1990; Kovacs, 1991; Marinelli et al., 1991; Schmidt et al., 1984; Vanhee et al., 1995a; Yang et al., 1999).

Cytokines: Tumor necrosis factor-α (TNF-α), TNF-α receptors (TNF-αR), and IL-1 IL-1 (α and β) and TNF (α and β) are two closely related cytokines sharing similar and synergistic effects with respect to inflammation and immunology. IL-1 and TNF-α are produced by activated macrophages

and have a wide spectrum of biologic actions on immune and nonimmune target cells. TNF-β is produced by activated T cells that bind the same receptor as TNF-α on target cells.

Mechanistic justification TNF-α is a proinflammatory cytokine, important in the early onset of inflammation, development, and progression of several diseases, including pulmonary fibrosis. TNF-α can be produced by a number of cell types including macrophages, monocytes, and polymorphonuclear cells (Dubravec et al., 1990; Rich et al., 1989). This cytokine interacts with two cell membrance-associated receptors to exert its effects (Bazzoni & Beutler, 1996; Smith & Baglioni, 1987), including recruitment of inflammatory cells (Driscoll et al., 1990a) and stimulation of cytokines, such as interleukin 1 (IL-1), chemokines, such as IL-8 and IL-6, and transforming growth factor beta (TGF-β) (Kunkel et al., 1990; Matsushima & Oppenheim, 1989; Pernis & Vigliani, 1982; Podor et al., 1989; Sime et al., 1998). In turn, the secretion of TNF-α is modulated by TGF-β (Chantry et al., 1989). A role for TNF-α in crystalline silica-induced fibrosis was suggested by in vitro studies with alveolar macrophages (Arcangeli et al., 2001; Dubois et al., 1989; Gosset et al., 1991), where this in vitro ability corresponded with the in vivo inflammatory activity of the crystalline silica (Driscoll & Maurer, 1991).

Interleukin-1 (IL-1) exists in biochemically distinct forms, called IL-1α and IL-1β (Cameron et al., 1986), where both appear to possess the same spectrum of biological activities and are recognized equally by IL-1 receptors (Dinarello, 1988; Kilian et al., 1986). IL-1 is produced by a number of cell types, including mononuclear phagocytes, polymorphonuclear leukocytes, and fibroblasts (Akahoshi et al., 1988; Gery et al., 1981; Tiku et al., 1986). It has been shown to play a crucial role in the process of fibrosis in the lung (Kolb et al., 2001).

Many in vitro experiments conducted on animal and human alveolar and peritoneal macrophages, as well as on peripheral blood monocytic cells, have shown that the stimulation of these cells by crystalline silica releases TNF-α and/or IL-1 (Bissonnette & Rola-Pleszczynski, 1989; Dubois et al., 1989; Gery et al., 1981; Gosset et al., 1991; Kampschmidt et al., 1986; Kang et al., 1992; Lassale et al., 1989; Lee et al., 1995; Lemaire & Ouellet, 1996; Lepe-Zuniga & Gery, 1984; Mohr et al., 1991; Oghiso & Kubota, 1987; Orfila et al., 1998; Savici et al., 1994; Schmidt et al., 1984; Seiler et al., 2001) through the upregulation of the TNF promoter (Savici et al., 1994).

Animal experiments in vivo have confirmed observations in vitro. For example, it was shown that, following exposure to crystalline silica, alveolar macrophages of exposed animals showed enhanced production of IL-1 (Oghiso & Kubota, 1986). A body of research on experimental animals has also concentrated on the proinflammatory cytokine TNF-α, which has been demonstrated to play a key role in particle-induced lung fibrosis and, more specifically, in quartz-induced lung fibrosis (Piguet, 1990; Piguet et al., 1990). Crucial experiments demonstrated that crystalline silica-induced lung fibrosis could be ameliorated using a specific anti-TNF antibody, and that the infusion of soluble-TNF receptors, which complex free TNF, could prevent and reduce existing fibrosis (Piguet, 1990; Piguet et al., 1990; Piguet & Vesin, 1994). These animal experiments and many others that followed (Driscoll et al., 1993, 1995; Sime et al., 1998) showed the importance of TNF in the cytokine network, leading to inflammation, tissue remodeling, and (interstitial) collagen synthesis (Ortiz et al., 2001).

Human studies for validation of the identified biomarkers of late response of inflammation, TNF-α, TNF-α receptors, and IL-1 Clinical evidence has demonstrated increased levels of IL-1, TNF-α, or TNF receptors in BAL cells, BAL fluid, lung tissue specimens, monocytes, or in plasma of subjects with various inflammatory lung diseases. For example, the spontaneous release of the two cytokines TNF-α and IL-1 by alveolar macrophages isolated from BAL fluid of CWP patients was found to be higher than that released by control cells (Lassale et al., 1989; Vallyathan et al., 2000; Vanhee et al., 1995a, 1995b). In addition, the levels of these cytokines were found to be elevated in the epithelial lining fluids of CWP patients (Vanhee et al., 1995a). The level of TNF-α in this same fluid of asymptomatic coal workers, on the other hand, was found to be lower, but there was no change in IL-1 despite the presence of crystalline silica particles and coal-mine dust in the BAL macrophages. This was attributed to lack of disease in these workers (Weber et al., 1996).

To evaluate the toxicity of coalmine dust from different regions, spontaneous release and crystalline silica-induced release of TNF-α were determined using blood monocytes isolated from

controls and CWP patients. It was found that the TNF-α release from workers correlated with dust exposure and the presence of disease with increasing radiological symptoms (Kim et al., 1999b; Lim, 1998; Porcher et al., 1994). A series of case-control studies have also shown that TNF-α release from blood monocytes could discriminate between coal miners with pulmonary and respiratory effects of coal dust (Borm et al., 1988; Jorna et al., 1994). Extensive epidemiological work has been published by Borm and colleagues, culminating in a 5-yr follow-up study to show the stability of this biomarker over the years, the effect of retirement on monocyte priming, and the predictive power of abnormal TNF release for 5-yr progression of CWP (Borm et al., 1988; Schins & Borm, 1995a, 1995b).

Serum TNF-α and soluble TNF-α receptors (p55, p75) were also assessed as biomarkers of CWP. Results suggested that serum levels of TNF receptors were associated with the fibrotic process of CWP and that serum cytokine levels may be correlated with the severity of CWP (Schins & Borm, 1995b; Zhai et al., 2002a). These latter studies, however, have contributed little value with regard to TNF release except perhaps as a potential effect modifier of free TNF-α.

Comments on the suitability of the biomarkers Overwhelming literature on in vitro and in vivo animal experiments, as well as epidemiological studies on human subjects, to validate inflammatory cytokines (especially TNF-α and IL1) makes these ideal biomarkers to assess exposure to crystalline silica or coal-mine dust, or to predict the progression of disease. Studies on TNF as a biomarker for CWP in coal miner cohorts from Belgium (Schins & Borm, 1995b) Germany (Morfeld et al., 2001), and France (Porcher et al., 1993) confirmed the validity of monocyte-derived TNF as a marker for CWP and, more specifically, in PMF. Schins and coworkers (1996) developed a whole-blood assay that is much easier to apply in occupational settings than that using monocytes and that delivers similar outcomes (Morfeld et al., 2001). In this assay system, as well as in isolated monocytes, the incubation time, when optimal for TNF, may not be so for other cytokines, and comparisons between different cytokines must be made at their optimal time points. The relative ease with which these measurements can be achieved (Schins et al., 1996) makes it all the more practical to consider them as biomarkers of choice in crystalline silica and coal mine dust exposures, and in CWP and silicosis.

Chemokine IL-8 and cytokine IL-6

Mechanistic justification Members of the chemokine family range in molecular weight from ~8 to 10 kD and possess a conserved 4-cysteine motif in their mature protein sequence. Chemokines are secreted by a variety of cell types, including fibroblasts, in response to IL-1 and TNF-α (Larsen et al., 1989; Nakamura et al., 1991; Rolfe et al., 1991; Standiford et al., 1991; Strieter et al., 1990) and are potent recruitment factors for neutrophils (Standiford et al., 1991). IL-6 is also produced by a number of cells, including monocytes and fibroblasts (Kotloff et al., 1990; May et al., 1988, 1989; Zitnik et al., 1993). IL-6 has different molecular sizes with main regulatory functions for B and T cells as well as in acute-phase responses (Ray et al., 1989; Van Dijk & Mackiewicz, 1995). As with IL-8, several cytokines, including IL-1 and TNF-α, are also known to stimulate the release of IL-6 (Ray et al., 1989). Therefore, a number of studies have been conducted to investigate the secretion of IL-8 and IL-6 in relation to crystalline silica exposure.

Human studies for validation of the identified biomarkers of late response of inflammation IL-8 was shown to be important in lung inflammation produced by crystalline silica. The expression of this chemokine was confirmed in BAL cells isolated from rats treated with an active from of quartz that produced inflammation, but not in BAL cells from rats treated with the same quartz sample, which was detoxified with aluminum to render it noninflammogenic (Duffin et al., 2001). Both TNF-α and IL-8 were found to increase in the supernatant of spontaneous or dust-stimulated monocytes isolated from peripheral blood and in sera of coal miners with pneumoconiosis (Kim et al., 1999b; Morfeld et al., 2001). The increase in IL-8 in human pulmonary epithelial cells in culture, following exposure to quartz, was also demonstrated via persistent upregulation of IL-8 gene expression in conjunction with NF-kappa B activation and I-kappa B-alpha depletion (Desaki et al., 2000; Schins et al., 2000).

Similarly, concomitant increases of TNF-α and IL-6 were demonstrated with human alveolar macrophages following exposure to coal dust and crystalline silica (Gosset et al., 1991). Upon stimulation with TNF-α, IL-6 was also found to be increased in lung fibroblasts isolated from patients

with silicosis (Arcangeli et al., 2001). The content of IL-6 in the BAL fluid was increased in CWP patients compared to controls (Lesur et al., 1994). The levels of this monokine in association with TNF-α were also increased in the supernatants of alveolar macrophages isolated from CWP patients as well as in lung biopsies, where mRNA of IL-6 was found to be limited to lung macrophages and associated with the presence of coal dust (Vanhee et al., 1995b). The increased levels of IL-6 in conjunction with TNF-α and IL-1 were also confirmed in BAL fluid of CWP patients and were correlated with the presence and progression of disease (Vallyathan et al., 2000). Serum levels of this monokine in CWP patients were found to be correlated, in conjunction with TNF receptors, with the severity of CWP (Zhai et al., 2002a).

Finally, in an attempt to elucidate the mechanisms involved in IL-6 induction by coal dust collected from different coal mines and its correlation to the presence of disease in these mines, the ability to stimulate kinase phosphorylation of AP-1 signal transduction pathways and production of IL-6 in both mouse epidermal JB6 and human epithelial A549 cells were investigated. In these two cell types, the increase in IL-6 involved both ERK and p38 MAPK pathways. In addition, the levels of IL-6 in both cell types treated with coal dust from three coal mine regions correlated well with CWP prevalence in these regions (Huang & Zhang, 2003).

Comments on the suitability of the biomarkers Despite evidence obtained from in vitro studies on the increased production of IL-8 and IL-6 following exposure to crystalline silica or coal-mine dust in macrophages and fibroblasts, only a limited number of human validation studies were reported in the literature. The increased levels of these two chemokines in BAL fluid of CWP patients, as well as the positive correlation between serum IL-8 and respiratory impairment found in a single human validation study, make these two chemokines potentially attractive biomarkers in silicosis and CWP as additional markers of inflammation, especially when measured concomitantly with TNF-α and IL-1.

Other neutrophil and monocyte chemoattractants: Macrophage inflammatory proteins (MIPs), cytokine-induced neutrophil chemoattractants (CINCs), monocyte chemoattractant protein (MCP), and arachidonic acid metabolites

Mechanistic justification Since inflammation and the recruitment of inflammatory cells play important roles in particle-induced fibrosis, chemokines and other biological macromolecules that may affect and control this process have been investigated in relation to silicosis.

Human studies for validation of the identified biomarkers of late response of inflammation Macrophage inflammatory proteins-1α and -β (MIP-1α and -β) are important chemokines in the recruitment of neutrophils in the lung and may contribute to the pathogenesis of inflammatory lung diseases (Driscoll, 1994; Driscoll et al., 1993, 1996). Their induction in alveolar macrophages, lung epithelial cells, and BAL fluid of animals upon exposure to crystalline silica is well documented (Driscoll, 2000; Driscoll et al., 1996, 2001; Yuen et al., 1996).

Cytokine-induced neutrophil chemoattractants (CINCs), with its four subtypes CINC-1, CINC-2 alpha, CINC-2 beta, and CINC-3 (CINC-1 being the major isoform), belong to the IL-8 family. In experimental silicosis, the presence of CINC-2-alpha, CINC-2-beta, and CINC-3 isoforms was reported with no detectable CINC-1 isoform in the cells. This suggested the importance of the first three isoforms as chemoattractants and in the formation of granulomas in chronic inflammation in pulmonary silicosis (Hata et al., 2003).

Eicosanoids are also well-known chemotactic molecules (Snyderman & Goetzl, 1981). In response to in vitro exposure to freshly fractured quartz, the levels of leukotriene B_4 (LT B_4), thromboxane A_2 (TX A_2), and prostaglandin E_2 (PG E_2) increased in human macrophages (Kuhn & Demers, 1992). In vivo exposure to crystalline silica increased the production of PGE_2 in alveolar macrophages of exposed animals (Henderson et al., 1991; Mohr et al., 1992). The production of platelet activating factor (PAF) by rat alveolar macrophages in response to crystalline silica and coal dust was also demonstrated (Lapp & Castranova, 1993).

The role of intracellular adhesion molecule-1 (ICAM-1) in leukocyte recruitment and adhesion in endothelial cells upon exposure to crystalline silica has also been investigated (Anderegg et al., 1997). Intratracheal administration of crystalline silica increased the levels of ICAM-1 in lung tissue and in BAL fluid, with concomitant influx of neutrophils to the lung (Nario & Hubbard, 1996).

A similar increase in ICAM-1 expression has been demonstrated in the lungs of coal miners, especially in bronchial and alveolar endothelial cells, with concomitant increase of its soluble shedding product, sICAM-1, in the lavage fluid of the lungs of the miners (Vanhee et al., 1996).

MCP-1, as a chemoattractant for monocytes (Rollins et al., 1991; Smith et al., 1995), was also studied and was found to be increased in BAL fluid and supernatants of alveolar macrophages isolated from BAL fluid and type II pneumocytes of patients with CWP (Boitelle et al., 1997).

Comments on the suitability of the biomarker The importance of these chemoattractants in perpetuating crystalline silica and coal dust-induced inflammation is well demonstrated in studies using BAL fluid from patients and lung cells in vitro. However, the limited number of studies and the lack of validation, as well as the difficulties associated with obtaining the biological samples, make the consideration of these macromolecules as biomarkers of choice in routine surveillance studies for silicosis difficult. This does not, however, preclude further evaluation of MCP-1 and ICAM-1 as biomarkers of silicosis.

Anti-inflammatory cytokine: interleukin-10 (IL-10)

Mechanistic justification IL-10 is reported to be an anti-inflammatory cytokine produced by a variety of cell types, including monocytes-macrophages. It has several functions, including reduced cellular recruitment of inflammatory cells in the lung with concomitant reduction of proinflammatory cytokines, such as TNF-α, MIP-1, and MIP-2 (Greenberger et al., 1995), with subsequent control of inflammation-related lung fibrosis (Kovacs & DiPietro, 1994).

Animal studies for validation of the identified anti-inflammatory biomarkers Two animal studies reported in 1998 confirmed the increase of IL-10 in cells obtained from BAL fluid and in lung tissue following exposure to crystalline silica (Driscoll et al., 1998; Huaux et al., 1998). This increase was accompanied by a decrease in inflammatory cytokines and decreased inflammation, but amplified fibrotic activity (Huaux et al., 1998).

Comments on the suitability of the biomarker Although IL-10 is an important biomarker in controlling inflammation and fibrosis, once again, the limited number of validation studies, as well as the difficulties in obtaining biological samples to determine its levels, makes it problematic for surveillance studies with crystalline silica and silicosis.

Lymphokines: interferon-γ (IFN-γ) and interleukin-4 (IL-4)

Mechanistic justification IFN-γ is a lymphokine with broad biologic functions, including an antifibrotic effect (Aggarwal & Behera, 2000; Gurujeyalakshmi & Giri, 1995) by antagonizing TGF-β, a fibrotic growth factor (Eickelberg et al., 2001; Wen et al., 2002). Although it is mainly produced by TH_1 lymphocytes, its production is also induced from AM by IL-12 and IL-18 in autocrine fashion (Keane et al., 2001; Munder et al., 1998). It is hypothesized that these two interleukins are initially produced by macrophages stimulated by crystalline silica, which then attract and activate lymphocytes to produce IFN-γ (Davis et al., 2001). IL-4, on the other hand, is produced by TH_2 lymphocytes, and its increased expression is associated with pulmonary fibrosis (Huaux et al., 2003).

Animal and human studies for validation of the identified biomarkers of pro- and antifibrotic lymphokines A persistent shift toward IFN-γ-expressing TH_1 cells within thoracic lymph nodes of silicotic animals has been demonstrated (Garn et al., 1997, 2000). The production of this lymphokine was also demonstrated in lung lymphocytes in silicotic animals (Davis et al., 1999, 2000), the gene deletion of which protected animals against crystalline silica-induced fibrosis (Davis et al., 2001). A single human study that assessed IFN-γ in BAL fluid of CWP patients indicated that this lypmhokine decreased in the alveolar spaces in these patients (Lesur et al., 1994).

Comments on the suitability of the biomarker From the limited work on IFN-γ and IL-4 in relation to inflammation and fibrosis, and the contradictory results regarding antifibrotic or profibrotic effects of IFN-γ, it is difficult to advocate their suitability as biomarkers of crystalline silica exposure or silicosis.

Growth factors: platelet-derived growth factor (PDGF), insulin-like growth factor type 1 (IGF-I), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and transforming growth factor (TGF)

Mechanistic justification Growth factors are low-molecular-weight polypeptides that may act on a variety of cell types and alter their proliferative and secretory properties (Patt & Houck, 1983;

Sime et al., 1998). Growth factors that have been indicated to be involved in fibrotic lung diseases include TGF, PDGF, bFGF, IGF-I, and EGF (Gauldie et al., 1993; Kelley, 1990; Krein & Winston, 2002; LeRoy et al., 1990).

Animal and human studies for validation of the identified biomarkers of fibrotic growth factors Biological samples tested for the levels of these growth factors included BAL cell fraction, BAL fluid, serum and blood cells, and lung tissues. For example, quantitative immunohistochemistry for mast-cell tryptase and for basic fibroblast growth factor (bFGF) in lung tissue from silicotic and control subjects indicated that mast cells were frequent in silicotic lungs compared to controls, which could produce bFGF and consequently may play an important role in the development of silicosis (Hamada et al., 2000).

BAL fluid in experimental animals and human subjects was also tested for the presence of these growth factors. BAL fluid or BAL alveolar macrophages from silicotic patients produced increased levels of growth factors (Li et al., 1992), such as alveolar macrophage-derived growth factor (AMDGF) (Rom et al., 1987). AMDGF was later discovered to be predominantly PDGF activity (Kumar et al., 1988; Shimokado et al., 1985), which had a proliferative effect on fibroblasts (Bauman et al., 1990). Other mediators identified from supernatants of alveolar macrophages isolated from BAL fluid of silicotic animals that had similar proliferative activity included IGF-1, PDGF, FGF, EGF, and TGF-β (Absher et al., 1993; Chen et al., 1994; Kumar et al., 1994; Lesur et al., 1992a; Melloni et al., 1994; Olbruck et al., 1998). This increased activity could be observed at the early stages of human and animal silicosis (Guoping et al., 1997; Lesur et al., 1992a, 1992b; Melloni et al., 1994, 1996). Treatment of crystalline silica-exposed animals with anti-PDGF antibody (Melloni et al., 1994, 1996; Ohta et al., 1997) and anti-bFGF (Liu et al., 1995b) significantly suppressed the development of pulmonary inflammation and fibrosis.

The spontaneous release of TGF-β by human alveolar macrophages, as well as release stimulated by coal dust and crystalline silica, was confirmed in CWP patients, where it was found to be significantly higher than those in control subjects (Schins, 1996). The same was found to be true with PDGF and IGF, in addition to TGF-β, in the same biological samples where high levels correlated with the severity of disease (Vanhee et al., 1994).

Finally, peripheral blood samples were tested for the levels of a number of growth factors in relation to disease. When the levels of PDGF-AA were measured in the supernatant of monocytes stimulated with or without coal dust, and in serum of coal miners with pneumoconiosis, no differences were seen (Kim et al., 1999b). Elevation of serum PDGF levels was detected in advanced silicosis patients. A positive correlation between PDGF levels with progression of disease indicated that this growth factor may be a reliable marker to monitor the progressive development and severity of silicosis (Brandt-Rauf et al., 1992).

Comments on the suitability of the biomarker Intuitively, PDGF and TGF-β should be reliable biomarkers, since their role in fibrosis is confirmed by key proof-of-concept studies such as the presence of TGB-β in histological sections of silicotic nodules and hyperplastic silicotic epithelium (Jagirdar et al., 1996; Williams et al., 1993; Williams & Saffiotti, 1995) and the ability of anti-PDGF and antisense PDGF DNA to downregulate the histopathological changes and collagen content of crystalline silica-exposed mouse lungs (Ohta et al., 1997). The role of these two growth factor cytokines is recognized as being central to the molecular pathogenesis of other types of fibrosis (Kelly et al., 2003; Zhang & Phan, 1996). Furthermore, TGF-β has been suggested as being anti-inflammatory and was found to be decreased in alveolar macrophages of subjects with PMF (Vanhee et al., 1994). Based on recent studies on the balance between TNF and TGF-β, and its relation to CWP (Borm & Schins, 2001), this hypothesis merits further epidemiological study. Both PDGF and TGF exist in different isoforms with largely different activities (Williams & Knapton, 1996; Zhuo et al., 2004), and TGF-β is activated in acidic conditions (Pellaud et al., 1999). These aspects need to be taken into consideration when using these growth factors as biomarkers of exposure to crystalline silica and silicosis.

Fibronectin

Mechanistic justification Fibronectin is one of the noncollagenous components of the extracellular matrix with a variety of functions (Limper & Roman, 1992; Yamada, 1989). For example, it is a macrophage-derived growth factor that stimulates the proliferation of lung fibroblasts (Bitterman

et al., 1983), acting synergistically with crystalline silica in stimulating collagen gene transcription in human 2BS fibroblasts (Liu et al., 1995a).

Animal and human studies for validation of the identified biomarkers of late response of fibrosis Spontaneous release of fibronectin by macrophages isolated from crystalline silica-exposed animals (Driscoll et al., 1990b) and in BAL fluid of CWP patients was higher than that of controls (Lassale et al., 1989; Rom, 1991; Rom et al., 1987). Fibronectin levels in the BAL of coal miners, on the other hand, were not elevated in asymptomatic subjects (Vallyathan et al., 2000; Weber et al., 1996). The observed increased plasma fibronectin in coal dust-exposed workers (Porcher et al., 1993) and in crystalline silica dust-induced diseases was thought to be related to enhanced predisposition to these diseases (Zhestkov, 2000).

Comments on the suitability of the biomarker Limited experimental and human validation data as well as its lack of specificity and sensitivity make it difficult to suggest fibronectin as a biomarker of effect for crystalline silica exposure and silicosis.

Carbohydrate antigen 19-9 (CA 19-9)

Mechanistic justification Carbohydrate antigen (CA) 19-9 is a cancer cell surface antigen, the elevation of which has been shown in the serum and BAL fluid of patients with a number of fibrotic lung diseases (Obayashi et al., 2000; Shimizu et al., 2002; Totani et al., 2001). CA19-9 may have neutrophil chemotactic activity and can be considered as a marker of inflammation. It may therefore play a role in the process of lung injury in patients with pulmonary fibrosis (Obayashi et al., 2000). The serum elevation of this marker in patients with pulmonary fibrosis has been considered as a negative prognostic factor (Fujita et al., 1998).

Human studies for validation of the identified biomarkers of lung injury Elevated serum levels of CA 19-9 were reported in a single silicotic patient. It was postulated that crystalline silica might stimulate lung epithelial-cell hyperplasia and hypertrophy and produce CA 19-9, raising serum levels. Immunohistochemical staining of autopsy specimens from the patient confirmed the presence of CA 19-9 in epithelial cells, which was correlated with the degree of destruction of the lung. Measurements of serum CA 19-9 were more sensitive than a chest radiograph in detecting lung fibrosis (Totani et al., 2000).

Comments on the suitability of the biomarker As indicated, this biomarker was reported in only a single silicotic patient. Many exposures that produce oxidant stress would also induce type II cell hyperplasia and hypertrophy. Therefore, the questions of specificity and sensitivity of CA19-9 for silicosis are raised, as the patient was already symptomatic.

Elastin

Mechanistic justification Elastin maintains the integrity of the lung by conferring the property of recoil to blood vessels, conducting airways, and alveoli (Hausladen et al., 1998). Its precursor, tropoelastin, is produced during a discrete period of development but not in adult animals (Pierce et al., 1995). Increased tropoplastin production is reported in a number of animal models of pulmonary fibrosis (Starcher et al., 1978).

Animal studies for validation of the identified biomarkers of lung injury In an animal study, crystalline silica-induced granulomatous inflammation was reported to accompany a substantial increase in the production of mature insoluble elastin. However, in situ localization of tropoelastin mRNA revealed that the expression of this gene was in (1) interstitial cells, (2) nonproliferating populations of α-smooth muscle actin-positive cells in the tips of alveolar septae, within the nongranulomatous areas, and (3) not in cells within granulomatous lesions (Mariani et al., 1995). Lack of tropoelastin in granulomatous lesions was attributed to the increased TNF-α accumulation in these lesions, which, in turn, may have modulated the expression of tropoelastin (Mariani et al., 1999). In addition, apparent lack of appreciable TGF-β staining in alveolar septae was considered as an indication that it is not responsible for increased tropoplastin production in silicotic lungs (Mariani et al., 1995), despite its known function as a stimulator of tropoplastin expression (McGowan, 1992b). On the other hand, an increase in elastin peptide in serum of coal-dust-exposed miners was observed, but this was shown to be an indication of altered elastin metabolism with no relation to the development of pneumoconiosis or its degree of severity (Porcher et al., 1993).

Comments on the suitability of the biomarker Based on the limited number of human validation studies, elastin may not be a suitable biomarker as its determination is invasive, requiring lung tissue. It is also nonspecific to silicosis, as elastin concentration was found to be inversely related to crystalline silica-induced granulomatous lesions. Therefore, serum elastin is not a suitable marker for the effect of crystalline silica due to its lack of relationship to the development of pneumoconiosis.

Collagen Synthesis/Degradation Network Collagen, elastic fibers, and proteoglycans are the main constituents of the connective tissue in the lung (McGowan, 1992a). Collagens are the most abundant molecules of the extracellular matrix and they are subject to continuous synthesis and degradation. Collagens are produced by a variety of lung cells, and a number of these collagens or components (collagen I, III, and VI) also have chemotactic activity on other cells (Laskin et al., 1986). Among collagen-producing cells, fibroblasts are the most important. However, collagen is also produced by epithelial and endothelial cells. Collagen production is controlled by a large set of cytokines, including TGFβ, TNFα, PDGF, fibronectin, and IL-1 (Mariani et al., 1996; Razzaque & Taguchi, 2003). Degradation of collagen is controlled by many enzymes, including collagenases, elastases, cathepsins, and gelatinase. Some of these, with indigenous controlling enzymes such as α_2-macroglobulin, α_1-antitrypsin, or tissue inhibitor of metalloproteinases (TIMP) (Garbisa et al., 1986; Hibbs et al., 1987; Mariani et al., 1998; Welgus et al., 1985), are, in turn, controlled by cytokines such as IL-1 and TGF-β (Overall et al., 1989). A complex network of factors is apparent, each with different kinetics and control on the synthesis/degradation rates of collagen in the lung. Since the amount and types of collagen determine the elasticity and physiological performance of the lung, the biological background of using this network to find and validate biomarkers for exposure or early effects of crystalline silica and silicosis is justifiable.

Procollagen type III N-terminal peptide (PIIIP)

Mechanistic justification PIIIP is a degradation product of type III collagen. It has been studied in relation to a number of lung diseases (Anttinen et al., 1986; Cavalleri et al., 1991; Luisetti et al., 1990), and its level in serum is believed to be a reflection of the current activity of an on-going deposition of collagen at sites of fibrosis (Gilligan et al., 1990; Pohl et al., 1992).

Animal and human studies for validation of the identified biomarkers of collagen synthesis/degradation Despite the relevance of collagen synthesis/degradation to silicosis, it is surprising that relatively few studies have been published on biomarkers arising from this field. A small set of studies has been published on the potential use of N-terminal propeptide of the pro-collagen type III (PIIIP), which can be detected in BAL fluid and serum. In an initial cross-sectional study, increased serum PIIIP was reported in coal miners with early-stage CWP (Janssen et al., 1992a). However, subsequent work on the same and extended study groups showed that PIIIP did not predict the progression of CWP over a 5-yr period and that its use as an exposure marker should be questioned (Schins & Borm, 1994; Schins et al., 1995a). Recently, PIIIP was found to be evaluated in combination with type VI collagen, as a parallel assessment of collagen synthesis and degradation (Schins et al., 1995a). This ratio was lower in healthy miners, suggesting that collagen accumulation is reduced in miners in the absence of disease.

Comments on the suitability of the biomarker Contradictory results and the lack of correlation between PIIIP levels and radiological progression of CWP, as reported in the limited number of validation studies, make PIIIP an unsuitable biomarker for predicting development or progression of pneumoconiosis.

Collagen-degrading enzymes

Mechanistic justification Matrix metalloproteinases (MMPs) are a group of structurally related endopeptidases that collectively digest most of the constituents of the extracellular matrix and basement membrane components. MMPs are classified into four major subgroups according to their domain structure and/or substrate affinity, including collagenases, stromelysins, gelatinases, and membrane-bound metalloproteinases. Those groups involved with collagen degradation include MMP-1, MMP-8, and MMP-13, which degrade fibrillar collagen, and gelatinases MMP-2 and MMP-9, which have substrate affinity for basement membrane type IV collagen (gelatin) and elastin (Birkedal-Hansen et al., 1993; Woessner, 1994).

Members of the tissue inhibitor of metalloproteinases (TIMP) family, that is, TIMP-1, TIMP-2, TIMP-3, and TIM-4, are capable of inhibiting the activities of all known MMPs and therefore maintain the balance between extracellular matrix deposition and degradation (Gomez et al., 1997).

Animal and human studies for validation of the identified biomarkers of collagen synthesis/degradation In an experimental lung fibrosis study, the activities of all matrix proteinases, collagenase 3 (MMP-13) and gelatinases A and B (MMP-2 and MMP-9), as well as the activities of TIMPs (TIMP-1 and TIMP-2), were increased in the lung tissues in early stages of silicosis. TIMP-1 and TIMP-2 showed a moderate reduction in the late stage of silicosis, suggesting extracellular matrix remodeling and basement membrane disruption (Pardo et al., 1999; Perez-Ramos et al., 1999). Additional studies of MMP-13 and its inhibitor TIMP-1 confirmed their involvement in matrix degradation via TNF receptors (TNFR) by altering the ratio of MMP-13/TIMP-1 expression in favor of degradation (Ortiz et al., 2001).

Complementary work has also been published on the activity of other relevant enzymes, which may play a role in regulating collagen synthesis and degradation, such as urokinase-type plasminogen activators (uPAs). These were shown to play a role in limiting fibrosis by disassembling the fibrin and procollagen and also by activating MMPs, the levels of which were shown to increase at the early stages of silicosis (Lardot et al., 1998). The levels of neutrophil neutral metalloendopeptidase elastase type (NMEP) and leukocyte elastase (HLE) were elevated in blood of coal miners (Porcher et al., 1993), confirming findings that coal dusts have potent effects in vitro on several elastases or antiproteases (Huang et al., 1993).

Comments on the suitability of the biomarker Further validation studies need to be performed for qualitative evaluation of all clinical work with collagen-degrading factors in relation to coal dust before one can consider any of the discussed biological molecules involved in collagen synthesis/degradation as practical biological markers for crystalline silica and/or silicosis.

Acute-Phase C-Reactive Protein (CRP) and Sialic Acid

Mechanistic justification CRP and sialic acid are two of the inflammatory markers reported to be increased in a number of inflammatory diseases (Crook et al., 1994; Crook et al., 1993; Gavella et al., 2003; Haq et al., 1993; Mendall et al., 1996; Ridker, 2003; Rothkrantz-Kos et al., 2003). Principal inflammatory cytokines, including IL-6, IL-1 TNF, IFN-γ, and growth factors TGF-β and EGF, affect the synthesis of acute-phase proteins, including CRP (Rokita et al., 1990). Induction of glycosylation of human CRP with sialic acid was also reported in different pathological conditions (Das et al., 2003).

Animal and human studies for validation of the identified biomarker The elevation of CRP and sialic acid in occupational lung diseases, including silicosis, was investigated as early as the 1950s and 1960s (Budanova & Danilova, 1965; Chiesura & Picotti, 1958; Gaultier et al., 1959; Ghislandi, 1958; Monteverde & Fumagalli, 1960; Rossi et al., 1962, 1965, 1967). The elevation of CRP in silicosis was correlated with the immunopathogenic effect of crystalline silica (Cojocaru et al., 1995; Fernandez Rego et al., 1991; Nigam et al., 1990). Later, sialic acid concentration and CRP were assessed in silicotic patients and were found to be positively correlated with the progression of disease (Cojocaru, 1997).

Comments on the suitability of the biomarker Although a number of studies have shown increased levels of serum CRP and sialic acid to be related to the degree of silicosis, these biomarkers are nonspecific and elevated in various types of inflammation and infection, and cardiovascular disease, as indicated earlier.

Apoptosis, sFas, mFas, FasL, and sFasL The role of crystalline silica-induced apoptosis of T lymphocytes was investigated in relation to immunological disorders. Apoptosis of phagocytic cells was investigated in relation to fibrosis.

Mechanistic justification Apoptosis (programmed cell death) plays many physiological roles (Ameisen, 1996; Thompson, 1995) and is associated with inflammation and fibrosis (Henson, 2003). It can be triggered by the binding of membrane-bound Fas (mFas) surface protein to its ligand (FasL) (Nagata, 1999). Several alternative spliced variants of the Fas gene have been reported, including the variant with the deletional form of exon 6 in the transmembrane domain

that is generally known as sFas. sFas protects cells from apoptosis by inhibiting membrane Fas (mFas)/FasL interaction by competition (Cheng et al., 1994; Ruberti et al., 1996). Fas ligand (FasL) is a membrane-bound and shed protein and the natural counterreceptor for the death-promoting Fas molecule expressed by a variety of lymphoid and nonlymphoid tissues. FasL is converted to its soluble form, sFasL, by a metalloproteinase-like enzyme (Hohlbaum et al., 2000).

Animal studies for validation of the identified biomarkers of apoptosis Crystalline silica-induced apoptosis has been proposed to contribute to crystalline silica-induced inflammation and fibrosis (Lim et al., 1999; Srivastava et al., 2002). Apoptosis of macrophages by crystalline silica (Leigh et al., 1997) via scavenger receptors was considered to be a critical factor in initiating an inflammatory response resulting in fibrosis (Iyer et al., 1996). Further confirmation of the involvement of apoptosis in crystalline silica-induced fibrosis of the lung was provided by animal studies that used Fas-ligand-deficient generalized lymphoproliferative disease mutant (*gld*) mice, which did not develop silicosis. Administration of neutralizing anti-Fas ligand antibody to wild-type animals in vivo blocked induction of silicosis, showing that FasL played a central role in the induction of pulmonary silicosis. It was therefore suggested that, as locally produced sFasL can induce bystander neutrophil apoptosis and block further neutrophil extravasation in mice, sFasL may play a role in modulation of crystalline silica-induced inflammation in humans (Borges et al., 2001).

Apoptosis of T lymphocytes, on the other hand, was investigated in relation to autoimmune diseases in silicotic patients. It was proposed that the elevation of sFas in serum suggests that the disruption of the competition between sFas and mFas to bind FasL in T-lymphocytes may be one of the most important mechanisms in the acquisition of autoimmunity in silicosis patients (Otsuki et al., 1998, 2000; Tomokuni et al., 1997, 1999). Factor analysis was also applied to evaluate new parameters related to Fas-mediated apoptosis, that is, membrane Fas expression on peripheral blood lymphocytes (mFas), serum sFas, serum sFasL levels, and the sFas/mFas mRNA expression ratios in peripheral blood mononuclear cells. The results clearly showed that these could be reliable tools for detecting immunological impairment independent of respiratory disorders in cases of silicosis (Otsuki et al., 1999). It was proposed that dysregulation of apoptosis in T lymphocytes may be correlated to predisposition of developing immunological disorders in silicotic patients (Tomokuni et al., 1997).

Comments on the suitability of the biomarker The finding of a central role of FasL in experimental silicosis is promising, as locally produced sFasL could induce bystander neutrophil apoptosis, reducing further neutrophil extravasation in mice. An important question is whether the role of FasL can be extended to modulation of crystalline silica-induced inflammation in humans. In conclusion, sFasL levels in serum may be a reliable biomarker of effect for silicosis and the levels of serum sFas/mFas (lymphocytes) may be a suitable marker for developing autoimmune diseases in silicotics. However, further human validation studies are needed to confirm the results from animal studies.

BIOMARKERS OF SUSCPETIBILITY

Biomarkers of susceptibility provide a means of assessing the variability of response by individuals to environmental stress, depending on the genetic makeup of the individual (Suk et al., 1996). A number of enzymes or other molecules exhibit polymorphisms, which may play a role in determining the extent and type of this response. Sequence variations in the genes encoding certain enzymes and other proteins may accumulate in a population. If the frequency of a specific variant reaches 1% or more in a population, it is referred to as polymorphism. Alternate genes containing these variants are known as alleles (Harris, 1980). The consensus is that interaction between susceptibility genes and exposure to a particular toxic substance may affect disease outcome (Greenberg, 1993). A number of cytokine polymorphisms have been associated with a variety of diseases (Yucesoy et al., 2003). Single-nucleotide polymorphisms (SNPs), that is, single-base-pair substitutions, represent 90% of all DNA sequence variations (Brookes, 1999). As of November 2003, 1.8 million SNPs have been discovered and identified (SNP, 2003).

Recently, the genetic influence of inflammatory response has been reviewed with the conclusion that, thus far, the strongest and most consistent association is with TNF-α, lymphotoxin-α, and

IL-1 receptor antagonist polymorphisms (Waterer & Wunderink, 2003). Biomarkers for genetic predisposition to a number of occupational diseases, including silicosis, have recently been reviewed (Spitsyn et al., 2000). Since inflammatory response features prominently in crystalline silica-induced fibrosis, TNF-α, lymphotoxin-α, and IL-1 receptor antagonist polymorphisms were investigated as obvious choices for genetic susceptibility of crystalline silica-exposed subjects to silicosis.

Interleukin-1 (IL-1) and IL-1RA polymorphism

Mechanistic justification IL-1 is a highly pleiotropic cytokine released primarily from activated monocytes or macrophages and many other cell types. Three genes located on the long arm of chromosome 2 encode for IL-1α, IL-1β, and IL-1 receptor antagonist (RA) (Roux-Lombard, 1998). Each of these genes possesses exonic polymorphisms resulting in changes in the cytokine expression, which is known to play an important role in certain inflammatory diseases (Blakemore et al., 1994; Clay et al., 1994; Tarlow et al., 1994). Studies have shown that IL-1 is involved in mechanisms that underlie the cascade of events leading to the development of pulmonary fibrosis, such as chemotaxis, inflammation, proliferation and secreation of connective tissue components, and various other steps promoted by it (Bussolino et al., 1986; Dunn et al., 1989; Ozaki et al., 1987; Raines et al., 1989). IL-1 appears to be intimately associated with the evolution of silicotic lesions (Davis et al., 1998).

Human studies for validation of the identified biomarker of genetic susceptibility Recent studies using lung tissues, which focused on differences in cytokine levels among individuals and inheritable SNPs contained within the regulatory elements of cytokine genes, support these observations in silicotic individuals. For example, the proportion of the IL-1 receptor antagonist (IL-1RA) (+2018) allele 2 genotype was found to be increased in miners with silicosis compared to control subjects. This was the first report to show an association between the observed polymorphism and increased risk for development of the disease (Yucesoy et al., 2001a). However, no significant or consistent differences could be seen in the IL-1α (+4845) or IL-1β (+3953) variants with respect to disease status (Yucesoy et al., 2001b).

Comments on the suitability of the biomarker Although there are no studies showing genetic associations between silicosis and cytokines, it is likely that IL-1RA polymorphism is a reliable susceptibility gene candidate; its variants may shed some light on the high incidence of silicosis and its severity in exposed populations. It is therefore considered to be a suitable candidate for biological monitoring in individual susceptibility to silicosis.

TNF-α Polymorphism

Mechanistic justification In several experimental studies, it was well documented that repeated cycles of macrophage injury and release of cytokines after crystalline silica exposure are major contributing factors in the development of silicosis. The strong link between overexpression of TNF-α during the genesis and progression of pulmonary fibrosis in humans exposed to crystalline silica and silicate minerals, as presented in a previous section, is well established. A number of studies also provide support for the involvement of a genetic component as a determinant of susceptibility and development of pulmonary fibrosis in animal and human models (Izmerov et al., 2002; Koskinen et al., 1983b; Kreiss et al., 1989; Ohtsuka et al., 1995). In humans, the gene encoding TNF-α is located on chromosome 6 between HLA-B and DR within the class III region of the major histocompatibility complex. Any changes, including SNPs, would therefore affect TNF-α production.

Human studies for validation of the identified biomarker of susceptibility The polymorphism in the TNF-α gene promoter, TNF2, was investigated in CWP. It was found that the TNF2 allele is associated with the development of large opacities in CWP (Kim et al., 2002). SNPs containing G → A substitutions have also been described in the promoter region at positions −308 and −238 in a number of other inflammatory pulmonary diseases (Huang et al., 1997; Schaaf et al., 2001; Whyte et al., 2000). A small case-control study on CWP patients and controls showed a significant association of disease with −308 TNF-α promoter allele, but since no relation was found between this genotype and the monocyte TNF phenotype, genotyping of TNF was considered less predictive (Zhai et al., 1998). In an earlier study, TNF phenotyping was found to be predictive for progression

of CWP (Schins & Borm, 1995a). A recent study of miners with moderate and severe silicosis showed that a minor variant, TFN-α (−238), was markedly higher in severe silicosis and significantly lower in moderate silicosis. It was also shown that, regardless of disease severity, IL-1RA (+2018) or TNF-α (−308) variants were elevated (Yucesoy et al., 2001a, 2001b). A study of South African gold miners showed that polymorphisms in the TNF-α gene promoter might predispose workers to severe silicosis. In this study, miners with severe silicosis were shown to have −238A and −376A alleles in linkage equilibrium. Severe silicotic patients were also reported to have increased mutation in the −308 allele (Corbett et al., 2002). These studies demonstrated reasonably high specificity and predictive value for TNF-α gene polymorphism if combined with other cytokines such as IL-1. TNF polymorphisms may therefore provide valuable information on disease susceptibility and severity in workers exposed to crystalline silica (Yucesoy et al., 2002).

Comments on the suitability of the biomarker Efforts were made to illustrate the utility of genetic information generated for minor variants in the genes coding for IL-1α, IL-1RA, and TNF-α as risk factors associated with increased susceptibility to silicosis. These studies suggest that gene–environment interactions are involved, with TNF polymorphisms, playing an important role in susceptibility and silicosis severity in exposed populations. Results of these analyses have, however, indicated that although this genetic information plays an important role in effectively characterizing risk groups, the presence or absence of these minor variants may not be a useful tool for individual classification (McCanlies et al., 2002).

Lymphotoxin Alpha (LTA) Polymorphism

Mechanistic justification TNF-α and lymphotoxin-α (LT-α) are related cytokines that are produced in response to oxidative stress or infection. Their genes are located adjacent to each other in the major histocompatibility complex class III region, on chromosome 6p21.3. TNF-α and LT-α act via the same two receptors, 55-kD TNF-RI and 75-kD TNF-RII. It was demonstrated that the surface expression of these receptors is necessary for the development of fibrosis in crystalline silica- and bleomycin-exposed TNF-α/LT-α gene double-knockout mice (Piguet et al., 1997).

Animal and human studies for validation of the polymorphism of the identified biomarker of susceptibility Recently, a hypothesis was tested to evaluate whether TNF −308 and LTA *Ncol* polymorphisms modify the pulmonary responses to oxidants in coal miners with different levels of exposure to cigarette smoke and coal mine dust. Overall findings showed severity of silicosis with TNF −308 at various stages of disease progression, as well as an association of CWP prevalence with *Ncol* polymorphism of LTA in individuals with low catalase activity. This confirmed the hypothesis of the interaction of gene and biological responses to oxidative stress (Nadif et al., 2003).

Comments on the suitability of the biomarker Based on available evidence, use of LTA *Ncol* polymorphism as a biomarker for silicosis is unproven.

MnSOD, GSTM1, GSTT1, and OGG1 Polymorphism

Mechanistic justification Evidence presented in earlier sections of this review suggests the involvement of reactive oxygen species and oxidative stress in CWP and silicosis, and the importance of the antioxidant enzymes of the exposed subjects to protect the individual against the ROS and oxidative stress. This may therefore justify investigations on polymorphisms of some of these antioxidant enzymes in relation to disease.

Animal and human studies for validation of the identified biomarker of susceptibility The polymorphisms of the antioxidant enzymes MnSOD, GSTM1, GSTT1, as well as the DNA repair enzyme OGG1, were investigated in CWP patients. No association was found between susceptibility to CWP and the polymorphism of these enzymes (Zhai et al., 2002b).

Comments on the suitability of the biomarker Use of polymorphisms of these antioxidant enzymes as biomarkers for silicosis based on available evidence is, at present, unwarranted.

Haptoglobin Polymorphism

Mechanistic justification Haptoglobilin (Hp) is an acute phase protein capable of binding hemoglobin (Hb) and forming a stable Hp–Hb complex, thereby preventing iron-induced oxidative

tissue damage (Asleh et al., 2003). Clearance of Hp–Hb is mediated through a macrophage scavenger receptor CD163. Hp also (1) acts as an antioxidant, (2) has antibacterial activity, and (3) plays a role in modulating many aspects of the acute phase response. Hp influences the T-lymphocyte function and specifically interacts with both resting and activated CD4+ and CD8+ T cells. Binding of Hp suppresses T cell proliferation. Hp inhibits Th2 anti-inflammatory cytokines, such as IL-1, IL-10, and IL-13, and plays a role in modulating proinflammatory cytokines, such as IL-1, TNF-α, IL-18, and IFNγ (Arredouani et al., 2003).

There are three major phenotypic forms, viz. Hp-1-1, Hp 2-1 and Hp2-2, associated with distinct clinical manifestations of different disease conditions of microvascular and diabetic complications (Ilzecka, 1996). Several studies demonstrated that functional allelic polymorphism in the Hp gene acts as a major determinant of susceptibility for the development of diabetic microvascular complications and is a risk factor for cardiovascular disease in individuals with diabetes (Hochberg et al., 2002). Hp polymorphism and its influence on iron metabolism in hereditary hemochromatosis are also well characterized (Delanghe & Langlois, 2002).

Human studies for validation of the identified biomarkers of susceptibility In studies on occupational respiratory diseases, seven highly polymorphic genetic variants, including Hp, were investigated by scientists in Russia. Five variants, including Hp, showed hereditary features of silicosis (Izmerov et al., 2002).

Comments on the suitability of the biomarker Use of Hp as a biomarker for silicosis, based on available evidence, is unwarranted.

DISCUSSION AND CONCLUSION

The literature reviewed shows that in vitro and in vivo animal studies have provided a basis for understanding the underlying mechanisms of the etiology of CWP and silicosis. Numerous biomarkers have been evaluated to assess effects following exposure to crystalline silica and/or coal-mine dust. Human validation studies substantiated some of these observations and argued in favor of their use as biomarkers for crystalline silica- and CWP-induced pneumoconiosis.

Based on these studies, the interaction of crystalline silica particles with phagocytic cells and the ensuing inflammation with the subsequent release of fibrogenic cytokines are considered fundamental to the understanding of the early events preceding fibrogenesis. Consequently, ideal biomarkers are those that could assess these early stages of interaction and inflammation prior to the onset of fibrosis. The stages identified, as well as the biomarkers involved, are summarized in a schematic presentation (Figure 2).

The aim of this review was to not only identify mechanistically justifiable biomarkers but also to identify those that could easily be used in routine human surveillance studies in different industries. As such, their relative ease of measurement, the biological material needed, and the invasiveness of the procedures were considered. A number of "ideal" biological markers were identified, namely, CC16 (serum), TNF-α (monocyte release), IL-8 (monocyte release), ROS measurement by chemiluminescence (neutrophil release), 8-isoprostanes (serum), total antioxidant levels measured by TEAC, glutathione, glutathione peroxidase, glutathione *S*-transferase, and PDGF (serum). In principle, these biomarkers could be assessed simultaneously by measuring their levels or activities in different peripheral blood fractions. Changes in the levels or activities of these biomarkers could then be used in the evaluation of dust-allaying measures in different industries or in the identification of subjects that may be susceptible or prone to developing silicosis.

A project is planned to test the validity and feasibility of these biomarkers to detect either early silicosis or susceptibility to silicosis in gold miners in South Africa. Baseline levels for the biomarkers will be measured and compared in different groups of miners, categorized according to cumulative dust exposure and disease status. A cohort of miners will be followed to determine changes in biomarker levels over time, as exposure changes and as disease develops and/or progresses. The biomarker levels will be correlated with lung function and radiography, as well as dust measurements.

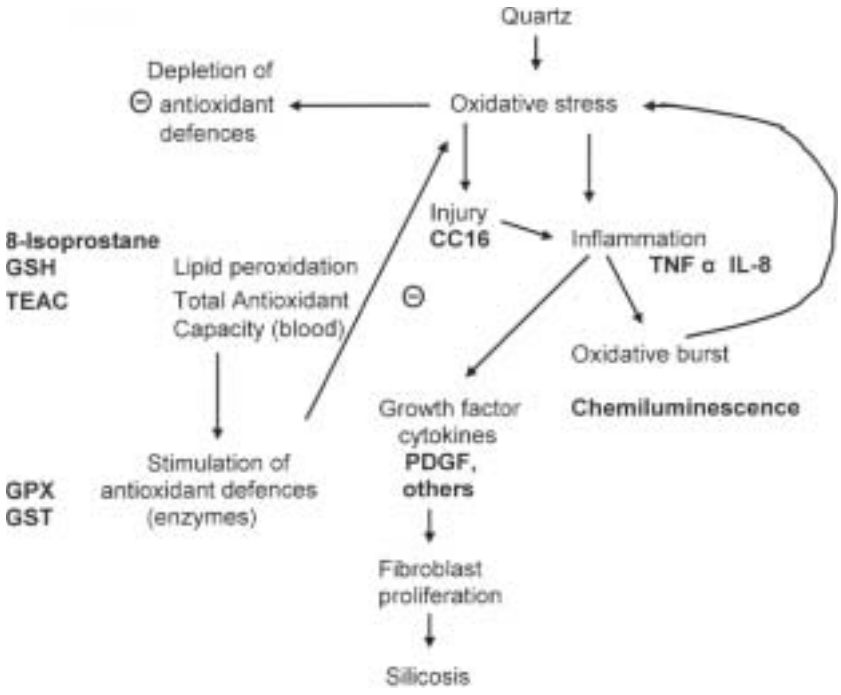

FIGURE 2. Schematic presentation of the mechanisms involved in crystalline silica-induced oxidative stress, injury, and fibrosis, with relevant useful biomarkers identified in these processes.

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