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REVIEW

Oxidant and antioxidant mechanisms of lung disease caused by asbestos fibres

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ABSTRACT: The pathogenesis of asbestos-related lung diseases is complicated and still poorly understood. Studies on animal models and cell cultures have indicated that asbestos fibres generate reactive oxygen and nitrogen species and cause oxidation and/or nitrosylation of proteins and deoxyribonucleic acid as a marker of cell injury. These effects are potentiated by the inflammation caused by the fibres. Recent studies have shown that individual variability in the antioxidant and/or detoxifying mechanisms probably has an important role in the development of asbestos-related lung diseases. Asbestos fibres cause both cell proliferation and apoptosis by multiple mechanisms, one of them being activation of signal transduction pathways by reactive oxygen and nitrogen species. Asbestos activates transcription factors such as nuclear factor kappa B, which has been shown to lead to the upregulation of antioxidant enzymes, most importantly manganese superoxide dismutase. This enzyme is also overexpressed in asbestos-related human malignant mesothelioma, whereas the induction of other antioxidant enzymes (copper-zinc superoxide dismutase, catalase, glutathione peroxidase) by asbestos fibres appears to be marginal. The significance of antioxidant enzymes in asbestos related diseases has, however, remained unclear. Furthermore, previous studies have not been able to offer successful therapies to the patients with asbestos-associated diseases. Only an improved understanding of the pathogenetic mechanisms in the human lung provides a basis for future therapies for asbestos-related diseases.

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Asbestos fibres play an important role in the pathogenesis of many occupational lung diseases, the most important of them being asbestosis, pleural effusion, pleural plaques and diffuse fibrosis, and malignancies, such as lung cancer and pleural mesothelioma. Occupational exposure to asbestos has decreased, but continues to be a reality, especially in the demolition of old buildings. The delay from the exposure to the development of asbestos-related lung disease is long, up to 20–40 yrs, which also explains why the incidence of these diseases is still increasing. Although much work has been conducted to investigate both the pathogenesis and treatment of asbestos-related diseases, many aspects are still poorly understood. Numerous studies have suggested the central role of free radicals in fibre-induced toxicity and their contributing role in asbestos-related lung diseases, but many of these studies have also yielded contradictory findings [1–8]. These controversies may be related to varying methodologies, the use of a wide range of fibre concentrations and differences in the animal and/or cell culture models used in various laboratories. The majority of the studies conducted on human asbestos-related diseases, such as mesothelioma, have addressed the clinical outcome and the effects of various therapies on the prognosis of these patients. These studies have not been capable in preventing the progression of these diseases or to cure these patients. This review will summarize the most important studies on the role of free radicals in asbestos-related cell and deoxyribonucleic acid (DNA) damage and the role of antioxidant defence against the damage caused by asbestos fibres and in asbestos-associated human lung malignancies.

The structure and classification of asbestos fibres has been extensively reviewed [7, 8]. Briefly, fibrous minerals can be classified into two major categories: asbestos and asbestiform fibres. The most important asbestiform natural fibres are wollastonite and talc, while the most important man-made fibres include glasswool, rockwool and ceramic fibres. Asbestos fibres, which will be discussed in this article, can be classified into two groups: serpentine fibres, the most important of them being chrysotile, and amphiboles, such as amosite, anthophyllite, crocidolite and tremolite. The physical and other properties of the fibres vary, which may also contribute to their toxicity. Short fibres are phagocytized more easily by macrophages and, therefore, cleared more efficiently from the lungs than long fibres which are incompletely phagocytized. Long fibres also cause more prominent generation of reactive oxygen species (ROS), and probably interfere with cell growth and division more efficiently than short fibres [5, 7, 8]. In addition to the differences in their physical properties, asbestos fibres also differ in chemical composition. All types of asbestos fibres contain variable amounts of iron cations as a part of their crystalline

structure. For example, amphibole asbestos, crocidolite and amosite, have an iron content of ~27%, whereas the iron content of serpentine fibre chrysotile is 1–6%.

The most important reactive metabolites in the pathogenesis of asbestos-related lung diseases are superoxide, hydrogen peroxide, hydroxyl radical and nitric oxide [7–12]. Superoxide anion, which is formed in one electron transport reactions from oxygen, is a relatively long-lived compound in biological systems. It is dismutated nonenzymatically and by superoxide dismutases to H_2O_2. H_2O_2 is less reactive than superoxide, but can exert its toxic effects by reacting with other molecules in producing hydroxyl radicals. Hydroxyl radicals can be formed directly from H_2O_2 and superoxide in the Haber-Weiss reaction or from H_2O_2 in the metal-catalyzed Fenton reaction. The reactivity of hydroxyl radical is very high, it is short-lived and reacts with its target molecule immediately. Since asbestos fibres have a high iron content, the pathogenesis of asbestos-related lung diseases has been suggested to be related to the metal-catalysed Fenton type reactions [7–15]. As reviewed elsewhere, many of these results have been controversial, and the iron content of different asbestos fibres does not necessarily correlate with fibre toxicity [7].

Other free radical-related mechanisms, especially those based on nitric oxide (NO), have been recently suggested to play an even more important role in asbestos fibre related toxicity than the metal-catalysed reactions. NO is a signal-transducing free radical which is synthesized from the guanidino nitrogen of L-arginine by nitric oxide synthase (NOS), the most important of them being constitutive and inducible NOS. Because NO is a free radical, it will react readily with other reactive oxygen metabolites, leading to the formation of toxic metabolites, most importantly peroxynitrite [16]. Peroxynitrite initiates iron-independent lipid peroxidation and oxidizes thiols much more efficiently than H_2O_2. In addition, peroxynitrite nitrates tyrosine and tryptophan residues in many proteins, and plays an important role in cell signalling, cell proliferation and cell death. NO and reactive nitrogen species (RNS) derived from NO have been shown to inactivate critical enzymes, cause DNA strand breaks, lead to the activation of the nuclear enzyme polyadenosine diphosphate-ribose polymerase (PARP) and inhibit DNA and protein synthesis [17, 18]. NO and its derivatives are toxic, not only *in vitro* but also *in vivo*, as has been shown in the lungs of hyperoxia or endotoxin-exposed rats, and in the lungs of adult respiratory distress syndrome (ARDS) patients (reviewed in [18]). These metabolites have also been shown to be produced in asbestos-exposed cells, which is why they may play an important role in the pathogenesis of asbestos-related diseases [18]. The most important reaction pathways of the reactive oxygen and nitrogen metabolites have been presented in figure 1.

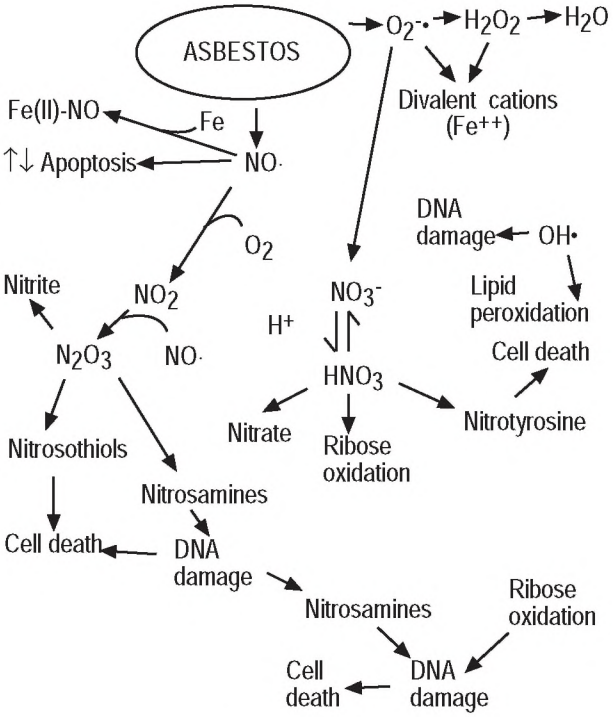

Fig. 1. – Pathways for the formation of reactive oxygen and nitrogen species by asbestos fibres. $O_2^{-}\bullet$: superoxide anion; OH•: hydroxyl radical; H_2O_2: hydrogen peroxide; NO•: nitric oxide; NO_3^-: peroxynitrite anion; HNO_3: peroxynitric acid; DNA: deoxyribonucleic acid. ↑: increase; ↓: decrease.

Generation of reactive oxygen and nitrogen species by asbestos fibres

High concentrations of asbestos fibres can generate ROS spontaneously in cell-free systems. When physiologically relevant concentrations of fibres and living cells are used, however, the generation of ROS by asbestos fibres appears to be variable. Most but not all studies have shown generation of ROS by inflammatory cells, but not in the target cells, such as pleural mesothelial cells, in response to asbestos fibres [7]. Most studies agree that asbestos fibres activate inflammatory cells to release reactive oxygen metabolites through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation *via* inositol phosphate and protein kinase pathways [19–22]. Possible reasons for the controversies of various laboratories include differences in the experimental conditions, such as cell species, cell isolation and culture, fibre types and concentrations, and the methods used in the assessment of ROS generation. For instance, long fibres cause a more pronounced elevation in the release of reactive oxygen metabolites by inflammatory cells than short fibres (reviewed in [7]). Undetectable ROS generation in the target cells may indicate that asbestos fibres do not cause any oxidant generation in those cells, or alternatively that ROS generation is undetectable since the reactive metabolites generated are rapidly consumed by intracellular antioxidants. Asbestos fibres generate nitrogen derivatives which cannot be detected by all the methods which measure superoxide or H_2O_2, which is why the generation of all reactive metabolites by asbestos is probably underestimated.

The most common methods used to asses ROS generation by asbestos have been based on the formation of stable spin adducts, such as 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO), or the detection of products of lipid peroxidation thiobarbituric acid-reactive substances and luminol-or luciferin-based chemiluminescence. The DMPO method has shown that asbestos causes hydroxyl radical generation in inflammatory cells [12, 23, 24], but this method appears

to be unable to detect free radical generation in human mesothelial cells exposed to asbestos fibres [25]. Asbestos causes lipid peroxidation especially in inflammatory cells [26, 27], which can be detected within the first 10 min of exposure [28]. It has to be emphasized that lipid peroxides represent the final damage from multiple reactions in the cell. Luminol or luciferin, which have usually been used in the chemiluminescence assay, detect a number of oxygen metabolites, which is why this method is a nonspecific assay of free radical generation [29]. Asbestos fibres cause a rapid increase in the chemiluminescence of human alveolar macrophages and polymorphonuclear leukocytes within the first few minutes of exposure, although not in human mesothelial cells [30–33]. Intact cells generate ROS and hydroperoxides when assessed by dichlorofluorescein-diacetate (DCFH-DA); this method is also able to detect a marginal increase in ROS generation by human mesothelial cells exposed to asbestos fibres [34]. DCFH-DA is freely diffusible and is oxidized to a fluorescent derivative by various oxidative reactions in the cells. Since direct measurement of ROS generation is difficult, and most methods are unspecific, ROS mediated reactions in cells exposed to asbestos fibres are still poorly understood.

One potential mechanism in causing free radical-related cell injury by asbestos fibres is the production of RNS. Isolated rat alveolar macrophages, when exposed to either crocidolite or chrysotile asbestos, have been shown to generate more nitrite than unexposed cells, which is indicative of increased NO production by asbestos fibres [35]. Recent studies also indicate that asbestos inhalation results in the production of nitrite species in rat alveolar macrophages, the production of which can be attenuated by N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of inducible nitric oxide synthase (iNOS) [36]. In agreement, asbestos fibres lead to the upregulation of iNOS messenger ribonucleic acid (mRNA) in alveolar epithelial (A549) cells [8]. Furthermore, experiments with the transfection of an iNOS promoter/luciferase reporter construct suggest that NO generation may be important in causing asbestos-related cell injury [36]. Recently, asbestos fibres were also shown not only to generate nitrite, but also to increase the immunoreactivity of nitrotyrosine, a marker of peroxynitrite formation, in the lungs of chrysotile and crocidolite exposed rats at 1 and 6 weeks of exposure [37]. In these experiments, nitrotyrosine was intensively stained at the alveolar duct bifurcations and within the bronchiolar epithelium, the alveolar macrophages and the visceral and parietal pleural mesothelium, whereas the lungs of sham-exposed rats demonstrated minimal immunoreactivity.

Activation of inflammatory cells by the fibres may play an important role both in the generation of ROS and RNS and in the development of asbestos-related cell injury. Rats exposed to crocidolite and chrysotile fibres show more pleural macrophages than sham-exposed animals, and the supernatants of these macrophages contain greater amounts of NO and tumour necrosis factor alpha (TNF-α) than those of control animals [37]. Thus, translocation of asbestos fibres to the pleural space contributes to the production of inflammatory cytokines and generation of toxic nitrogen radicals. These results are in agreement with a recent finding that NO generation by alveolar macrophages correlates with the amount of neutrophils in bronchoalveolar lavage samples after asbestos inhalation [36], and that interferon gamma (IFN-γ) synergistically enhances the formation of RNS induced by the fibres [35]. Another recent study also indicated that interleukin (IL)-1β enhances the upregulation of iNOS mRNA levels and the formation of nitrite in rat mesothelial cells exposed to asbestos fibres [38]. These findings strongly suggest the role of inflammation and inflammatory cytokines in potentiating the generation of RNS during asbestos exposure. The same is probably true of other ROS, since superoxide generation by inflammatory cells, but not by pleural mesothelial cells, is enhanced by asbestos fibres, and cell injury of mesothelial cells cultured in the presence of inflammatory cells can be attenuated by antioxidants [39, 40]. However, no conclusions can be made as to the relative importance of ROS and RNS in the development of asbestos-related lung injury. Furthermore, the significance of RNS in the pathogenesis of human asbestos associated lung diseases still remains unclear.

Hardly any studies are available on the free radical generation by cells derived from asbestos-related malignancies. ROS generation by malignant cells might have an important role in both cell proliferation and tumour resistance. In one recent study, ROS generation was clearly less abundant in the mitochondrial compartment of mesothelioma cells than in nonmalignant mesothelial cells [41]. This finding was associated with a highly elevated activity of mitochondrial manganese superoxide dimutase (MnSOD), an enzyme that plays a central role in decomposing superoxide radicals in the cells [42, 43]. Further studies are needed to clarify the role of ROS and RNS generation in asbestos-related nonmalignant and malignant cells and disease progression. ROS generation may have a potent role in cell survival, apoptosis and tumour progression.

Deoxyribonucleic acid damage caused by asbestos fibres

Oxidant exposure results in DNA single strand breaks, oxidative DNA lesions such as 8-hydroxy-2-deoxyguanosine (8-OHdG), depletion of cellular high-energy nucleotides and, finally, either apoptotic or necrotic irreversible cell damage, or cells with DNA alterations which may serve as progenitor cells for malignant conversion [44, 45]. As reviewed previously, numerous studies have suggested that asbestos fibres generate hydroxyl radicals which may then lead to the formation of 8-OHdG [7]. Many previous results have implicated the importance of iron and the Fenton reaction in the development of these DNA lesions, since fibres can bind Fe(II), a natural form of iron, in biological material. However, recent data have further indicated that iron-free fibres also produce DNA breaks and guanine hydroxylation, suggesting that other mechanisms may be responsible for the formation of DNA damage by asbestos. These recent studies using human lung epithelial (A549) cells have indicated that crocidolite causes formation of 8-OHdG and also that aminoguanine (AG), an inhibitor of NOS, reduces the formation of nitrite by these cells and prevents 8-OHdG formation [46]. Thus, RNS may have an important role in the development of DNA damage caused by the fibres.

Amosite or crocidolite exposures have caused DNA strand breaks in some but not all studies [7, 39, 47–49]. This inconsistency may be related to the sensitivity of the assay procedure, since DNA strand breaks in asbestos-exposed cells have been investigated using many different methods. In several studies, asbestos fibres did not cause DNA strand breaks when assessed by the DNA unwinding biochemical technique (reviewed in [49]), whereas lactate dehydrogenase (LDH) release, which is a marker of lytic cell injury, was significantly enhanced in asbestos and oxidant-exposed cells [39]. Other possible factors which may contribute to the observed differences in DNA breaks include fibre type, the wide variation in the concentrations of fibres, duration of exposure and the existence of various cell types with different potential in developing asbestos-induced DNA damage. For instance, mesothelial cells have been shown to be more sensitive than epithelial cells when assessed by a colony-forming assay [50], and transformed human mesothelial cells (MeT-5A) appear to be more resistant than rat mesothelial cells in primary culture [51]. The mechanisms explaining this difference, however, remain unclear.

COMET assay (single cell gel electrophoresis) is a new and sensitive method, which has recently been used in the assessment of DNA breaks in human airway epithelial and pleural mesothelial cells exposed to oxidants and asbestos fibres [34, 52]. A recent study showed that exposure to crocidolite (1–4 $\mu g \cdot cm^{-2}$) for 4–72 h causes a marginal increase in the mean tail moment of human mesothelial cells indicative of DNA strand breaks [34]. The effect of asbestos fibres in causing an increase in the mean tail moment was significantly smaller than the corresponding effect caused by a sublethal concentration of H_2O_2. In these same conditions, TNF-α, which has been shown to be crucial in the development of interstitial lung fibrosis in transgenic animals [53] and important in asbestos related inflammation [54], caused minimal increase in the mean tail moment, which was further potentiated by asbestos fibres [34].

Previous studies have mainly been conducted with animal and cell culture models. The effects of asbestos fibres *in vitro* are not directly applicable to the situation *in vivo*, where the concentration of the fibre is often lower but may persist for many years. It can, however, be hypothesized that both the inflammatory potential of asbestos fibres and reactive oxygen and nitrogen metabolites play an important role in the pathogenesis of human asbestos related malignant diseases.

Apoptosis caused by asbestos fibres

The mechanisms of cell death include necrotic and apoptotic pathways. A decrease in the normal apoptotic response may be implicated in oneogenesis since cell death contributes to the removal of damaged DNA and thereby preventing tumour promotion. These mechanisms are complicated and may be different in various cells and tissues and in the animal and human lung. Apoptosis may also be modulated by multiple mechanisms, although one ot the most important mechanisms is the generation of free radicals and/or a change in the cellular redox state. Typical features in apoptosis are the activation and cleavage of cysteine proteases known as caspases and the development of DNA fragmentation and cell death, so that the plasma membrane remains intact in contrast to the lytic cell injury, where the cell membrane is damaged. The central contributing factor in apoptosis is the release of cytochrome c from the mitochondria, which activates caspases by binding to Apaf-1 and triggering caspase-9 activation leading to a proteolytic cascade that culminates in apoptosis (reviewed in [55]). The most important mitochondrial proteins modulating apoptosis are the Bcl2 family of proteins (*e.g.* Bcl2, BclXl, bax). The dimerization of these proteins may either inhibit (*e.g.* Bcl1-Bcl2-dimer) or promote (*e.g.* bax-dimer) the release of cytochrome c from the mitochondria. Cell death and apoptosis may also proceed in the absence of mitochondria and even in the absence of caspases, many of these reaction pathways being still poorly understood [55, 56].

Asbestos fibres have recently been shown to cause apoptosis by an unclefined triggering mechanism. Asbestos fibres can cause dose-dependent apoptosis in rat, rabbit and human pleural mesothelial cells [57, 58]. Chrysotile and crocidolite cause apoptosis in human alveolar macrophages within 48 h of exposure [59]. Bronchiolar and alveolar epithelial cells from rats exposed to asbestos show apoptosis in contrast to its rare occurrence in normal rat lung [60]. Since apoptosis caused by asbestos has been shown to be ameliorated by exogenous antioxidants, catalase, superoxide dismutase and desferrioxamine, free radicals have been suggested to play a role in asbestos-induced apoptosis [57, 58]. One important enzyme which is induced during oxidant stress is PARP, an enzyme which participates in DNA repair. Oxidants, but also asbestos fibres, lead to PARP activation indicative of oxidant-related DNA damage [61]. Rabbit pleural mesothelial cells undergo apoptosis when exposed to crocidolite (5 $\mu g \cdot cm^{-2}$) so that ~15% of the fibre-exposed cells are apoptotic, the corresponding value in control cells being 4%.

Apoptosis induced by asbestos can be inhibited not only by extracellular catalase and hypoxia (8%) but also by 3-aminobenzamide, an inhibitor of PARP, which also suggests the importance of free radicals as a mechanism in asbestos-induced apoptosis [62]. Given that only a small percentage of cells undergo apoptosis due to asbestos, the effect of asbestos fibres on cell proliferation [63] may play a critical role in contributing to tumour promotion by asbestos fibres in the lung. This is also in agreement with the recent results showing that asbestos fibres stimulate many signal transduction pathways which regulate cell survival and proliferation [63].

The evidence on the role of ROS and RNS in asbestos-related DNA and cell damage is summarized in table 1.

Effect of antioxidants on asbestos-related cell injury

Indirect markers of free radical effects on cells include the protective effect of iron chelators, exogenous antioxidants and inhibitors of NOS on the development of cell damage caused by fibres. Desferrioxamine has been found to reduce free radical generation, DNA strand breaks and cell damage caused by asbestos fibres *in vitro* in many but not all investigations [7, 14, 64, 65]. Antioxidants such as glutathione, *N*-acetylcysteine (NAC) and liposome or polyethyleneglycol (PEG)-entrapped antioxidant enzymes,

attenuate asbestos-related toxicity in a variety of cells [7, 11, 66–68, 77]. Furthermore, transfection of the MnSOD gene ameliorates asbestos-mediated cytotoxicity in hamster tracheal epithelial cells [69]. One of the basic studies conducted *in vivo* demonstrated that the administration of PEG-conjugated catalase through a pump mechanism inhibited the development of crocidolite-induced pulmonary inflammation and interstitial fibrosis in rat lungs [68]. Most of these studies are either inhalation studies with experimental animals or *in vitro* studies with cultured animal cells. Amosite-induced cell injury of human mesothelial cells in the presence of fibre-activated neutrophils can also be attenuated by H_2O2 scavengers, most significantly by catalase [33, 70, 78]. However, asbestos-induced toxicity is not limited to free radical injury alone, since the deleterious effects of asbestos fibres have been shown to be decreased by antioxidants in some, but not all investigations (reviewed in [7, 49]). Asbestos fibres not only contribute to ROS- and RNS-related cell injury but also cause inflammation, a phenomenon which cannot be abolished by exogenous antioxidants.

Antioxidant enzymes in cells exposed to asbestos fibres

Since free radicals have been considered a potential pathogenetic mechanism in asbestos-related pulmonary diseases [1–4], one possibly important determinant in the development of lung injury caused by fibres is the antioxidant capacity of the cells. Mammalian tissues contain three types of superoxide dismutases (SODs), which scavenge superoxide to H_2O2 and protect the lung against high oxygen tension [79, 80]. These SODs are MnSOD in the mitochondria, CuZnSOD in the cytosol [81] and extracellular (EC)SOD in the extracellular matrix [82]. The most important H_2O2-scavenging enzymes are glutathione peroxidases (GPx) and catalase. Intracellular GPx is localized mainly in the cytosol of epithelium [83], and extracellular GPx functions at least in the epithelial lining fluid of the airways [84]. γ-glutamylcysteine synthetase (γ-GCS) is the rate-limiting enzyme in glutathione synthesis [9]. Glutathione-S-transferases (GSTs) consist of a superfamily of phase II metabolic enzymes that catalyse the conjugation of reduced glutathione with an electrophilic group of a wide range of compounds. In general, the reactions which are catalysed by GSTs protect cells against cytotoxic and carcinogenic agents. GST isoenzymes can be classified into four major classes, α, μ, θ and π, and their composition varies in different tissues. Numerous GSTs, most importantly GST-π, have been detected in human lung [85]. In addition to these enzymes, the epithelial lining fluid of the airways contains low molecular weight antioxidants, one of them being glutathione [86, 87].

Table 1. – Direct and indirect evidence for the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by asbestos fibres

Detection of hydroxyl radicals and chemiluminescence in inflammatory cells [12, 23, 24, 30–33]
Production of superoxide and H_2O_2 in inflammatory cells [19]
Generation of RNS by macrophages, epithelial cells and mesothelial cells [35–38]
Activation of inducible nitric oxide synthase [8, 38]
Detection of lipid peroxidation [26–28, 47]
Protective effects of iron chelators and exogenous antioxidants [11,14, 64–70]
Activation of signal transduction and transcription factors [71, 72]
Downregulation of signal transduction by antioxidants [71, 73]
Induction of DNA strand breaks and production of 8-hydroxydeoxyguanosine [45–48, 50–52]
Activation of polyadenosine diphosphate ribose polymerase (PARP) [61]
Induction of apoptosis [57–59, 62]
Induction of protective mechanisms (superoxide dismutase, haeme oxygenase) [74–76]

DNA: deoxyribonucleic acid.

Reactive oxygen metabolites and cytokines induce antioxidant enzymes in the lungs of experimental animals and in cells derived from various animal tissues, one of the most important of these enzymes being MnSOD. This enzyme is induced by changes in the cellular redox state, by inflammatory cytokines, such as TNF-α, interleukins and IFN-γ [87–96], and by high oxygen tension in the lungs of hyperoxia-exposed animals [97–101]. MnSOD appears to be induced by the same cytokines, but not by hyperoxia in cultured human airway epithelial cells [94, 102, 103]. The induction of other antioxidant enzymes in human lung by oxidants and cytokines has been variable and usually less extensive than the induction of MnSOD [74, 102–105].

Asbestos fibres induce antioxidant enzymes, the most important of these enzymes again being MnSOD [75, 76]. The results obtained with asbestos fibres have been parallel in animal and human cells and suggest that the effect of asbestos fibres on antioxidant enzyme (AOE) expression is less intense than the effects of cytokines, such as TNF-α [51, 76, 106]. JANSSEN *et al.* [74] compared the induction of the MnSOD gene in cultured human pleural mesothelial cells and human lung fibroblasts exposed to crocidolite or chrysotile asbestos, a nonfibrous analogue of crocidolite asbestos, riebeckite and xanthine/xanthine oxidase (X/XO). The steady-state mRNA levels of MnSOD were elevated in cells exposed to asbestos and X/XO, and the induction of MnSOD was similar for crocidolite and chrysotile asbestos. Both fibres and X/XO caused a prominent increase in the mRNA of MnSOD, but the change in the level of immunoreactive protein remained only modest. The same study indicated that nonfibrous riebeckite does not cause induction of MnSOD. Various cells may respond differently to asbestos fibres; the asbestos fibres did not cause any increase in the mRNA or protein of MnSOD in fibroblasts in contrast to mesothelial cells, whereas X/XO induced gene expression of MnSOD in both cell types [74]. *In vivo* inhalation of fibrogenic nonasbestiform minerals by rats showed that inhalation of cristobalite and ultra-fine TiO_2 a particle causing pulmonary inflammation, caused significant increases in the mRNA levels of MnSOD, whereas noninflammatory nonfibrogenic minerals failed to induce MnSOD [75]. Thus, asbestos fibres cause significant induction of MnSOD, but its magnitude appears to be related to the inflammatory response caused by the fibres.

The effects of asbestos fibres on other AOEs, such as CuZnSOD, GPx and catalase, have been nonexistent or less intense than the effect observed with MnSOD [75, 105, 107]. In addition, exposures to fibrogenic or nonfibrogenic nonasbestiform fibres do not cause significant

induction of these AOEs [75]. Thus, inflammation plays a less evident role in causing induction of CuZnSOD, catalase or GPx either alone or in combination with asbestos or other mineral fibres.

Haeme oxygenase (HO) has antioxidative properties, since it converts haeme to biliverdin, which means that the haeme pool is reduced. Haeme is known to function as a pro-oxidant. HO has been shown to be upregulated by oxidant stress, which is why this enzyme can also be used as an indirect marker of the oxidant effects on cells. HO is induced by crocidolite and chrysotile asbestos fibres in human pleural mesothelial cells, but as is the case with MnSOD, the induction may be cell-specific since asbestos fibres do not cause any induction of HO in human lung fibroblasts [74].

Transcription factors and oncogenes in cells exposed to asbestos fibres

Nuclear factor kappa B (NF-κB) is a transcription factor associated with the induction of numerous genes during inflammation and lung defence. Some of the most important genes which contain NF-κB-regulatory elements in their regions include those for iNOS and MnSOD. Activated NF-κB is bound to specific DNA sequences leading to the synthesis of selected enzymes in oxidant-mediated inflammation. Asbestos fibres activate NF-κB, which activation has been shown to lead to the induction of MnSOD and iNOS in asbestos-exposed cells [71]. For instance, exposure of hamster tracheal epithelial cells to crocidolite asbestos induces activation of NF-κ B-dependent genes and these reactions can be modulated by the antioxidant NAC [71], which confirms the role of reactive metabolites in the activation of signal transduction by asbestos.

The *c-fos* and *c-jun* oncogenes are early response oncogenes which encode proteins of the Fos and Jun families. Activation of these genes is associated with increased binding of transcription factor AP1 to the DNA of the promoter region of a number of genes closely associated with cell proliferation. Both crocidolite and chrysotile asbestos cause dose-dependent increases in the expression of *c-fos* and *c-jun*, at least in rat pleural mesothelial cells [72]. The induction of these proto-oncogenes is cell and species-specific. For instance, asbestos fibres induce *c-jun* but not *c-fos* in hamster tracheal epithelial cells. The expression of either of these genes is not altered after exposure to nonfibrous and biologically inactive analogues of asbestos or to inert particles [72]. The elevation of the levels of *c-fos* and *c-jun* oncogene expression by asbestos fibres can be partly prevented by antioxidants, such as NAC [73]. Furthermore, exposure of rat pleural mesothelial cells to asbestos causes depletion of cellular glutathione, a response that can be abolished with NAC. Also, pretreatment of cells with buthionine sulfoximine, an agent which causes glutathione depletion by inhibiting the rate limiting enzyme in glutathione synthesis (γ-GCS), increases the induction of *c-fos* and *c-jun* mRNA by asbestos [73]. On the other hand, the exogenous oxidants H_2O2 and xanthine oxidase do not alter the mRNA levels of *c-fos* or *c-jun*. Thus, the signalling pathways leading to *c-fos*/*c-jun* proto-oncogene induction by asbestos are not triggered directly by extracellular ROS. It appears that the intracellular thiol levels (*e.g.* glutathione and NAC) contribute to the expression of *c-fos* and *c-jun*, suggesting a redox-sensitive component in the signalling cascade modulating the gene expression of *c-fos* and *c-jun* by asbestos. The activation of these oncogenes is closely associated with cell proliferation and may therefore play a central role in the free radical-associated carcinogenesis caused by asbestos fibres.

Since the activation of various transcription factors may lead to the induction of antioxidant enzymes in cells and tissues, these induction mechanisms might play an important role in the protection of cells against tumour promotion, but also in the induction of these mechanisms in the tumours caused by asbestos fibres.

Despite intensive investigations by several laboratories, the importance of proto-oncogenes in asbestos related malignancies have remained unclear. ROS and RNS generated by the fibres may have effects on the tumorigenicity by inactivation of tumour suppressor genes such as *p53*. However, most studies suggest that the frequency of *p53* gene mutation is not directly related to asbestos exposure (reviewed in [5]).

Antioxidant enzymes in human asbestos-related lung tumours

Very little is known about the expression of AOEs in lung diseases associated with asbestos exposure. One important determinant in predisposing individuals to oxidant-induced malignancies is probably genetic susceptibility and the individual variation in the levels of various AOEs. Only GST has been investigated for its genetic variability and risk for asbestos-induced malignant diseases [108, 109]. Ten-fold interindividual variation in GST activity has been reported both in normal and malignant tissues, which appears to be related to genetic variability. Polymorphism

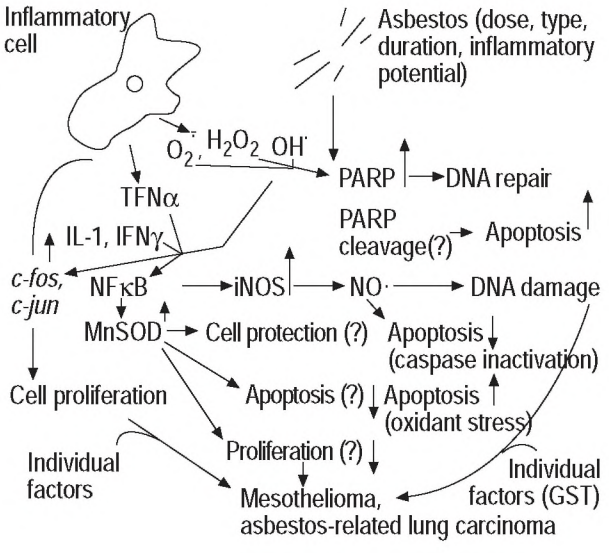

Fig. 2. – Possible mechanisms contributing to the pathogenesis of asbestos-related lung diseases. For abbreviations see figure 1. PARP: adenosine diphosphate ribose polymerase; NF-κB: nuclear factor kappa B; iNOS: inducible nitric oxide synthase; MnSOD: manganese superoxide dismutase; TNF-α: tumour necrosis factor alpha; IL: interleukin; IFN-γ: interferon gamma; GST: glutathione-S-transferase. For remaining definitions, see footnote to figure 1.

of GST-μ has been shown to be associated with an increased risk for lung cancer [110, 111]. In addition to GST, several other metabolic enzymes are associated with metabolizing exogenous compounds, one of them being *N*-acetyltransferase (NAT). Recent studies have shown associated NAT2 genotype in individuals exposed to high levels of asbestos fibres enhance their susceptibility to asbestos-related lung disorders [109, 110]. Furthermore, these individuals with GST-μ null genotype in combination with slow acetylation-associated NAT2 genotype have about a two-fold risk of developing malignant mesothelioma. No other studies are available on the polymorphisms of antioxidant enzymes (such as MnSOD) and the consequent risk to develop asbestos-related lung diseases. The individual variability in the AOEs and genetic factors combined with the level of exposure probably have a significant role in the development of asbestos-related lung diseases.

Previous studies have suggested that one of the most important antioxidant enzymes, MnSOD, is a tumour suppressor gene [112–115]. Most of these studies were conducted on malignant cells transfected with the MnSOD gene and showed suppression of the malignant cell type and decreased cell viability of the cells or tumours with high MnSOD levels. The situation is, however, different *in vivo*, where multiple AOEs may be induced simultaneously. The mRNA level, immunoreactivity and specific activity of MnSOD have been shown to be high in human malignant mesotheliomas and cell lines compared to normal healthy pleural mesothelium or nonmalignant mesothelial cells [42, 43]. In addition, many groups have documented high MnSOD immunoreactivities in other malignant tumours as well [116–119].

JANSSEN *et al.* [119] have recently reported that high MnSOD was associated with poor overall survival of patients with colon carcinoma. This finding is in agreement with the role of MnSOD in protecting cells and tissues against endogenous and exogenous free radicals. Resistance to high oxygen tension and oxidants appears to correlate with SOD induction or high MnSOD levels in cells [70, 73, 74, 83, 120]. Various cytotoxic drugs are also known to cause activation of NF-κB, which can then lead to the induction of MnSOD [121, 122]. Recent studies have, however, shown that SOD induction or transfection without simultaneous induction of other AOEs antioxidant enzymes may provide minimal or no protection at all [123], or may even be harmful, leading to increased H_2O_2 generation and enhanced cell injury and apoptosis [96, 124, 125]. The balance between the different AOEs is probably more important than the activity of any single enzyme. Rat and human airway epithelial cells, mesothelial cells, and vascular endothelial cells, for instance, consume exogenous oxidants by multiple mechanisms [126–130]. The importance of multiple mechanisms is also supported by the finding that MnSOD induction caused by amosite asbestos fibres does not offer any protection during subsequent oxidant or fibre exposure [51, 106]. The same is probably also true of human mesothelioma, which is extremely resistant both to radiation and chemotherapy. One important issue in this disease is whether the resistance of this tumour is associated with the high levels of MnSOD, coordinated induction of multiple AOEs or other mechanisms which can prevent cell death such as cell proliferation or apoptosis by nonoxidant mechanisms. So far, the data for these other mechanisms are limited. Mesothelioma cells are resistant cells, but the expression of several antiapoptotic proteins, such as Bcl2, in these cells is also low or undetectable [131].

The levels and activities of other AOEs in human mesothelioma cells are variable. For instance, resistant mesothelioma cell lines appear to contain not only high levels of MnSOD, but also significantly elevated activities of catalase and GST and elevated glutathione concentrations [43, 132]. Among four mesothelioma cell lines investigated, the most resistant mesothelioma cell line contained elevated MnSOD, catalase, GPx and glutathione [132]. Furthermore, the depletion of glutathione in these cells made them vulnerable to exogenous oxidants and the cytotoxic drug epirubicin [132].

GST-π expression has been considered to be one preneoplastic tumour marker. Elevated levels of GST in the tumour may be associated with primary drug resistance or the enzyme may be induced during various treatment modalities (reviewed in [133]). In human mesothelioma, nearly all non-neoplastic mesothelial biopsies have been shown to be positive for GST-α and GST-π and about half of non-neoplastic cases have been positive for GST-μ [134]. GST-μ and GST-π expression also correlate significantly with survival [108, 134]. A recent study conducted on lung cancer (not asbestos-related) indicated low catalase, GPx and GST immunoreactivities in most of the nonasbestos-related lung tumours investigated [83]. γ-GCS has been shown to be upregulated at least during chemotherapy in some malignant tumours [135]. Recently, the mRNA of the γ-GCS heavy subunit was shown to be overexpressed in four or five cultured mesothelioma cell lines, whereas it was undetectable by polymerase chain reaction in one human mesothelioma cell line [136]. No data on the expression of this enzyme *in situ* are available. The activity of γ-GCS correlates with intracellular glutathione. Glutathione is required not only in the reaction of glutathione peroxidase, but also in the reaction of GST with its substrates and in the glutathione-dependent multidrug resistance pump on the plasma membrane. One recent study has addressed the expression of P-170 glycoprotein in malignant mesothelioma, and in that particular study immunohistochemical staining with the P-170 glycoprotein antibody indicated that nearly all mesothelioma biopsies were positive for this glycoprotein [136]. These previous findings suggest the importance of glutathione metabolism in the resistance of mesothelioma cells not only against oxidants but also against cytotoxic drugs *in vitro* and possibly also *in vivo*.

Conclusions

Asbestos fibres generate ROS and RNS most prominently in inflammatory cells. The mechanisms of asbestos-induced DNA damage are unclear, but probably associated with the combined oxidant effects of asbestos fibres and fibre-induced inflammation (fig. 2). Very little in known about the genetic susceptibility in developing asbestos associated diseases, but recent data such as results with GST, suggest that individual variability in the antioxidant and detoxification mechanisms play a role in the development of asbestos-related lung diseases. The induction of pulmonary defences, such as the induction of MnSOD, by

cytokines and asbestos fibres, is well elucidated, but whether the inducibility of various AOEs protects cells or prevents carcinogenesis is still unclear. Asbestos fibres also induce apoptosis and cell proliferation. Only a small proportion of the cells undergo apoptosis, which may explain why apoptosis caused by the fibres does not prevent tumour promotion. Asbestos associated human mesothelioma has elevated expression of AOEs, most importantly MnSOD, but the significance of the expression of various AOEs in the prognosis of these patients remains unclear.

Previous and recent findings have failed to prevent or cure asbestos-related diseases. More studies are needed to explore the individual risk factors for asbestos-related lung diseases, mechanisms of cell proliferation and apoptosis caused by asbestos, and the effects of various antioxidant mechanisms on the resistance of malignant lung cells against chemotherapy and radiation.

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