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Predisposing Factors in Occupational Lung Cancer: Inorganic Minerals and Chromium

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Reactive oxygen species (ROS) have been implicated in the pathogenesis of cancer. Inhalation of inorganic minerals such as asbestos and crystalline silica, and metals such as arsenic, beryllium, chromium, nickel, and vanadium, may promote directly and indirectly enhanced generation of ROS at a persistent level in concert with chronic inflammation. Perpetual ROS generation can cause specific molecular changes resulting in the activation or inactivation of transcription factors that may alter gene expression leading to cell proliferation, differentiation, and carcinogenesis. The mechanisms involved in the signal transduction leading to these processes are the subject of intense investigation. In this review, some of the recent findings from our laboratories concerning key molecular events elicited by asbestos, crystalline silica, and chromium are presented. These include genotoxicity, DNA damage, lipid peroxidation, activation of transcription factors activator protein-1 (AP-1) or nuclear factor kappa B (NF-κB), and *p53* or k-*ras* gene alterations. From these studies, it is evident that ROS signaling is critical for the responses of cytokines, growth factors, and activation or inactivation of transcription factors that promote carcinogenesis.

KEY WORDS: occupational cancer, lung cancer, cancer epidemiology, inorganic minerals, chromium, reactive oxygen species.

Introduction

Occupational and environmental exposure to certain types of mineral particles can cause lung cancer and other diseases. The mechanisms by which inorganic particles induce carcinogenesis are not known. Recent studies from our laboratories and others have postulated that reactive oxygen species (ROS) may play a pivotal role in the initiation and promotion of carcinogenesis.[1–6] The association with chronic inflammation is well recognized for many types of cancer development in humans. In animal experiments persistent inflammation caused by nongenotoxic particulate overload phenomena is implicated in the development of lung tumors in rats.[7,8] Enhanced generation of ROS at a persistent level, combined with this ongoing inflammation, can cause specific injury to molecular targets resulting in carcinogenesis.

ROS that are generated by multiple pathways have been shown to be involved as downstream mediators of micromolecular changes that contribute to several stages in carcinogenesis.[1,2] The regulation of gene expression, changes in the signal transduction cascade, and alteration in apoptosis and *p53* are redox sensitive and are known to be involved in carcinogenesis.

Inactivation or activation of specific oncogenes results in genomic changes in the DNA, leading to mutations, which are important in

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carcinogenesis. Central molecular events in the carcinogenic process are caused by mutations in certain oncogenes. From the data available on carcinogenesis, it might be possible to estimate that approximately 10 to 20 mutations may be required for the development of cancer. We and others have shown that inhalation of insoluble particulates such as asbestos, coal, crystalline silica, and other inorganic minerals may stimulate ROS and cytokines play a crucial role in inflammatory mechanisms. Persistent ROS generation may lead to inactivation or activation of the molecular events and promote carcinogenesis.[9–15]

Bronchogenic carcinoma is the most common lung neoplasm in males in the United States, and it is increasing dramatically in females. The contribution of occupational and environmental factors to the development of lung cancer is incontrovertible. Percival Pott in 1775 was the first to report the incidence of an excess of scrotal carcinomas among young chimney sweeps in the United Kingdom.[16] Since then several occupational and environmental factors have been implicated as direct human and /or animal carcinogens or pro-carcinogens. Doll and Peto[17] estimated that ~15% of the lung cancer in men and ~5% in women could be attributed to occupational exposures. The current magnitude of occupationally related lung cancer in the United States is estimated to be about 10%.[18] The exact proportion of lung cancers caused by occupational and environmental factors is difficult to ascertain. This is basically due to mixed exposures containing carcinogens and pro-carcinogens involved in exerting synergistic or additive interactions. Furthermore, cigarette smoking is the most important etiologic factor and information on smoking history is often biased.

Because many occupational and environmental cancers are caused by multiple agents and several pathogenic mechanisms are involved in carcinogenesis, an understanding of the molecular and biochemical pathways involved in the development of neoplasm by the inhalation of asbestos, crystalline silica and metal ions, such as chromium, may provide insights into some of the potential pathways believed to be important in inorganic mineral-induced carcinogenesis. This review briefly addresses some of the molecular features elicited by asbestos, crystalline silica, and chromium that are considered important in the carcinogenic process.

Asbestos

Asbestos is a versatile product, with useful qualities such as fireproofing, strength, and durability. It is used in thousands of industrial applications and is an occupational hazard. Asbestos is also recognized as a health hazard in the general environment because it pervades the ambient air during mining, industrial applications, and demolition of buildings containing asbestos products.[19–21]

Asbestos-associated carcinogenesis has been the subject of several epidemiologic, pathological, analytical and in vitro and in vivo experimental studies.[22] It is estimated that ~24% of deaths among workers heavily exposed to asbestos is due to lung cancer.[22–24] Cigarette smoking enhances the carcinogenic properties of asbestos ~10 to 11-fold compared with nonsmoking asbestos workers.[22–24] Mesothelioma, a signal neoplasm of asbestos exposure, was almost unknown before the extensive use of asbestos. The incidence of mesothelioma is ~8% in asbestos workers. Cigarette smoking does not seem to be associated with an increased risk of mesothelioma in an asbestos-exposed population.

The properties of asbestos fibers are very important in the tumorogenic potential. In comparison with fibers less than 5.0 μm in length, long fibers have greater carcinogenic potential. The fiber length combined with bio-durability of certain types of asbestos (crocidolite, amosite) suggests that these types of asbestos function as complete carcinogens to the mesothelial cells producing mesothelioma. Erionite fibers, similar in many respects to amphibole asbestos, are also known to produce mesotheliomas in humans and experimental animals. In studies evaluating the carcinogenic properties of man-made mineral fibers, ceramic fibers with a high bio-durability have been found to be carcinogenic in animal exposure studies.[25,26] It was estimated that between 1968 and 1990 a total number of 28,000 asbestos-related cancer deaths and 12,000 malignant mesothelioma deaths occurred in the United States.[27]

Crystalline Silica

Crystalline silica was suggested as a carcinogen in 1985.[28] In 1986 a working group organized by the International Agency for Research on Cancer (IARC) concluded that there was ample evidence for the carcinogenicity of silica in animal studies, while in humans it was considered only as a

probable carcinogen.[29] In 1997 IARC convened another working group and concluded that there was sufficient evidence in humans for the carcinogenicity of silica.[30] However, debate still exists concerning the conclusive nature of studies on humans and the wide variability of effect among animal species. Therefore, we continue to investigate some of the molecular mechanisms involved in silica-induced carcinogenesis.

Mechanisms Involved in Carcinogenesis by Asbestos and Crystalline Silica

Inflammation

Chronic inflammation may predispose to fibrogenesis and carcinogenesis because both processes may have common mechanistic pathways involving growth factors and cellular proliferation. Inflammation is a distinct hallmark of mineral dust inhalation and generally is associated with focal or diffuse inflammation at sites where inhaled particulates are present in the lung. Acute or chronic inflammation is frequently associated with the exposure to toxic fibrogenic particulate such as asbestos and crystalline silica, whereas a transient inflammation is observed with nontoxic dusts such as barite or iron oxide. Persistent inflammation is accompanied by recurring oxidative stress. Inhaled toxic, durable particles such as asbestos and crystalline silica are shown to enhance production of ROS through four different mechanisms:

1. Particles may contain iron/trace metals or redox sites with a potential to generate OH radicals through Fenton or Haber-Weiss reactions.
2. Physical characteristics, toxicity, and biodurability of the particulates may promote frustrated phagocytosis resulting in sustained generation of ROS.
3. Inflammatory cells elicited by the exposure to these particles may generate high levels of ROS.
4. Finally, generation of ROS will overwhelm natural antioxidant defenses and initiate a vicious cycle of inflammation, ROS generation, and its interaction with DNA, cellular constituents, and repeated injury.

Genotoxicity

Genotoxic agents generally produce damage or constitutional changes in the DNA and chromosomes. Asbestos and crystalline silica are generally considered nongenotoxic in standard bioassays. Asbestos is nongenotoxic in several mutation assays, such as the Ames Salmonella assay, bone marrow chromosomal aberration, and micronucleus assays. However, asbestos causes deletion mutations in hamster-human hybrid cell lines and other abnormalities in mesothelial cells through the influence of ROS generated from the surface of asbestos.[31–35]

Similar to asbestos, crystalline silica produces no apparent changes in standard genotoxic assays. In cell transformation assays, crystalline silica, in concentrations similar to chrysotile asbestos, induced a dose-dependent increase in cell transformation frequency.[36–40] Hesterberg and Barett reported a dose dependence of asbestos and crystalline silica in the transformation of mammalian cells in culture. On the other hand, intraperitoneal injection of crystalline silica did not induce micronuclei changes in mice bone marrow. It is evident from a literature review that the genotoxicity studies using crystalline silica are conflicting. A direct interaction of particulate with the genetic material during cell division is implicated in the mediation of changes observed.

Carcinogens are generally grouped according to their ability to produce dose-dependent genotoxicity in short-term bacterial mutagenesis assays, micronucleus assays, cytogenetic assays, and nongenotoxic cell proliferation assays.[36–40] Nongenotoxic or epigenetic carcinogens are generally negative in short-term genotoxicity studies. It is believed that they cause changes in gene expression without any major alterations in genetic material. Several epigenetic carcinogens are known to function as promoters of carcinogenesis. Although the mechanisms involved in the epigenetic pathway are unclear, it is generally believed that activation or inactivation of oncogenes modulated by ROS and cytokines play a pivotal role in cell proliferation leading to preneoplastic and neoplastic transformation.

Another mechanism by which the epigenetic carcinogens influence cell differentiation and proliferation is through the up-regulated enzymes such as ornithine decarboxylase and protein kinase C.[41,42] Nongenotoxic chemical carcinogens are also known to enhance activation of enzymes that are important in tumor promotion and differentiation.

DNA Damage

Studies reported by Daniel et al.[39] showed oxidative changes to DNA, possibly caused by the pro-

ductions of ROS in proximity to DNA, inducing oxidation of base and thymol glycol. These studies suggested that direct damage to DNA occurs with crystalline silica generated ROS. We and others have detected DNA strand breaks to cells and phage λ Hind III DNA using the alkaline unwinding assay.[1] Silicic acid, leached from crystalline silica, is considered to be responsible for these changes by some investigators.[43] However, in our studies freshly fractured quartz, which is characterized by a significant increase in Si and Si-O radicals, produced a twofold increase in DNA strand breaks.[1] It has been shown that in aqueous media silicon-oxygen-based radicals react in the presence of metal ions to produce highly reactive species, such as hydroxyl radicals (·OH). ·OH radicals are known to cause DNA strand breaks, point mutations, chromosomal aberrations, and alterations in purine and pyrimidine bases leading to genetic damage.[44]

Severity and significant differences observed in DNA strand breaks among the different types of asbestos containing varying concentrations of iron and with fresh or aged crystalline silica were reported.[1] Oxidative damage to DNA, specifically by ·OH, is a common phenomenon. A major product of this attack on DNA is 8-hydroxy-2′deoxyguanosine. Using asbestos and crystalline silica in the presence of DNA, we also showed significant differences between asbestos and crystalline silica in the extent of oxidative damage to DNA.[1]

Activation of Transcription Factors: Activator Protein-1 (AP-1) and Nuclear Factor Kappa B (NF-κB)

It is becoming increasingly clear that ROS are important signaling molecules that may mediate different functional responses in cells or tissues. Activation of numerous signaling cascades is mediated by ROS, including the family of mitogen-activated protein kinases (MAPKs). Activation of these kinases leads to the phosphorylation of nuclear targets, including protein subunits of transcription factors. This mediates the expression of many classes of genes. Activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) are two key factors known to be under ROS control.[45,46]

Both asbestos and crystalline silica stimulate MAPKs through ROS generation resulting in activation of AP-1 and NF-κB transcription factors in cell culture models, as well as in animal models.[2,54] AP-1 transcription factor consists of homo/heterodimer of Fos and Jun protein family members. It binds to DNA at the promoter regions of a number of intermediate genes governing inflammation, proliferation, and apoptosis. In cell culture systems, expression of both c-*fos* and c-*jun* is required for transition of the cell cycle from G_1 to S phase.[47] Fos and Jun proteins are considered immediate early response gene products that regulate the expression of other genes required for progression through the cell cycle.[47] Overexpression of c-*jun* is observed in early stages of lung cancer.[48] Up-regulation of AP-1 activity by asbestos and silica in the lung may be critical to the pathogenesis of both pulmonary fibrosis and lung cancer.

NF-κB resides in the cytoplasm and after activation by a number of stimuli, including cytokines, ROS, and pathogens. It is translocated to the nucleus. NF-κB is a highly regulated transcription factor linked to activation of a number of genes that contain NF-κB-binding sites in their promoter or intronic regions. These genes, which include genes encoding various interleukins and nitric oxide synthase and the protooncogene c-*myc*,[49–51] may be intrinsic to cell proliferation and inflammation, which are essential features of silica- and asbestos-induced carcinogenesis as well as other pulmonary diseases.

Using the mouse macrophage cell line RAW 264.7, Chen et al.[53] in our laboratories have demonstrated that crystalline silica is a potential inducer of NF-κB activation. Catalase blocks silica-induced NF-κB activation, while superoxide dismutase, which converts $O_2^{\cdot-}$ to H_2O_2, exhibits an opposite effect, that is, increasing silica-induced NF-κB activation. Metal ion Fe(II) enhances NF-κB activation, whereas the metal chelator deferoxamine reduces NF-κB activation. These results indicated that crystalline silica mediates ·OH radical generation via a Fenton-like reaction and that ·OH radicals may be involved in the mechanism of silica-induced NF-κB activation.

The signal transduction pathways leading to transcription factor activation by asbestos have been studied extensively in our laboratories using in vitro as well as in vivo systems.[54] In transgenic AP-1 reporter mice, exposure to asbestos caused a time-dependent activation of AP-1 that persisted for 72 hours to 10-fold in the lung tissue and 22-fold in bronchial tissue.[54] Recently we have also demonstrated that crystalline silica activates AP-1 in a stable AP-1-luciferase reporter plasmid-transfected JB6 P+ cell line and in AP-1 luciferase reporter transgenic mice. In in vitro as

well as in vivo studies we have observed that freshly fractured crystalline silica is able to induce AP-1 activation to a greater extend than aged silica. In these studies, H_2O_2 and O_2^- were identified to be responsible for silica-induced AP-1 activation, thereby confirming the central role of ROS in the activation of this important transcription factor in carcinogenesis. The role of AP-1 complexes with c-*jun* and c-*fos* in cell proliferation and transformation is well recognized.[54–57]

p53 *and* k-ras *Gene Alterations*

Activation of protooncogenes or inactivation of tumor suppressor genes are often associated with carcinogenesis. Molecular alterations are frequently present in lung cancer induced by certain etiologic agents. In lung cancer, *k-ras* and *p53* have been shown to mutate at greater frequency in response to an etiologic insult. Cigarette smoking and uranium exposure are known to cause specific molecular changes in the *p53* and *k-ras* genes.[58] Liu et al[59] presented an analysis of *p53* and *k-ras* mutations in asbestos- and silica-related lung cancer cases. The mutational spectrum appears to be distinctly different in lung cancer cases of workers exposed to silica where adenocarcinoma had a *p53* mutation rate of 54% in contrast to the usual lung cancer mutation rate of 70% in squamous cell lung cancer cases. They also reported an increased frequency of *k-ras* mutations in codons 13 and 15 with G-C transversion, instead of the usual G-T transversion in lung cancer.

Carcinogen/Tumor Promoter

Asbestos appears to be a complete carcinogen to mesothelial cells in humans and experimental studies.[11,15,24] Asbestos produces chromosomal aberration, aneuploidy, and mitotic interference in mesothelial cells as a result of its direct penetration and interaction with the cells. On the other hand, tracheobronchial epithelial cells are resistant to these changes, so asbestos probably promotes carcinogenesis in these cells as a co-carcinogen.[11,24,60,61] Molecular alterations in oncogenes and tumor suppressor genes in human mesothelioma cases and animal experimental studies are well characterized, but are not observed in asbestos-induced lung carcinomas.[24,60]

Asbestos and crystalline silica are capable of inducing ornithine decarboxylase and protein kinase C in hamster tracheal epithelial cells.[41,42] Ornithine decarboxylase is important in the biosynthesis of polyamines that are required in cellular replication, while protein kinase C activation is associated with cell proliferation. Protein kinase C is an intracellular mediator for tumor promoters and may play a crucial role in cell-signaling pathways involved in the regulation of early response genes. Both of these enzymatic markers respond to a dose-dependent induction, which is similar to classic tumor promoters.[24]

Chromium

Chromium-containing compounds are widespread in soil, sediments, and ground water.[62,63] Epidemiological, animal, and cellular studies have established that Cr(VI) compounds are toxic and carcinogenic.[64,65] Cr(VI) and Cr(III) states differ in their biologic activity. Cr(VI) is actively transported into cells by an anionic transport system, while Cr(III) does not easily penetrate cells and is not easily oxidized by cellular constituents.[66] As Cr(III) does not interact with DNA in the absence of reducing agents, it is generally believed that reduction of Cr(VI) to lower oxidation states is an important step in chromium-induced carcinogenesis.[67,68]

Reduction of Cr(VI)

Intensive studies have been carried out to examine the formation of reactive chromium intermediates during the reduction of Cr(VI). A multiplicity of mechanisms are responsible for reduction of Cr(VI) to Cr(III), depending on the nature of reducing agents. Cr(V) formation has been observed in the reduction of Cr(VI) by various reducing agents, such as ascorbate,[67] glutathione,[69] and NADPH-dependent glutathione reductase.[67] Cr(V) has also been observed in the reduction of Cr(VI) in intact cells.[70] NADPH-dependent reductases are likely to be the major species responsible for Cr(V) formation. Using electron spin resonance (ESR) with a low-frequency microwave bridge and cylinder shape loop gap resonator, we were able to show that a Cr(V) intermediate can be observed following the one-electron reduction of Cr(VI) in whole living animals.[71–73] This generated Cr(V) was found predominantly in the liver with a small amount in the blood. This Cr(V) species was identified to be a Cr(V)-NADPH complex with an oxygen bond to Cr(V).

Generation of Free Radicals from Chromium Reactions

Using ESR spin trapping, glutathione-derived thiyl radical ($GS^{\cdot}$) was detected in the reaction

between Cr(VI) and glutathione.[69] The thiyl radicals generated may cause direct cellular damage. These radicals may react with other thiol molecules and molecular oxygen leading to the generation of superoxide O_2^- radical. The addition of H_2O_2 to a mixture of glutathione and Cr(VI) generated hydroxyl ·OH radicals. The ·OH radical generation was due to the reaction of Cr(V) with H_2O_2 via a Fenton-like reaction:[74].

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + {\cdot}OH + OH^-$$

Cr(V), Cr(IV), Cr(III), and possibly Cr(II) are also able to generate ·OH radical through Fenton-like reactions as described above.[75] O_2^- radicals are capable of causing one-electron reduction of Cr(VI), Cr(V), and Cr(III). Thus, Cr(VI), Cr(V), and Cr(III) can generate ·OH radicals via Haber-Weiss reaction as described below.

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {\cdot}OH + OH^-$$
$$M^{(n+1)+} + O_2^- \rightarrow M^{n+} + O_2$$

Overall,

$$O_2^- + H_2O_2 \xrightarrow{M^{n+}/M^{(n+1)+}} O_2 + {\cdot}OH + OH^-$$

It may be noted from the above reactions that several oxidation states of chromium are able to generate ·OH radicals via Haber-Weiss reactions. Because these chromium ions function as Haber-Weiss catalysts, a large amount of ·OH radicals can be generated if O_2^- and H_2O_2 are available. The Haber-Weiss mechanism of ·OH generation could be particularly significant during phagocytosis subsequent to exposure to Cr-containing respirable particles. In this process, macrophages and other pulmonary cells generate large quantities of O_2^- and H_2O_2 during the so-called "respirable burst".

Role of Free Radical Reactions in Cr(VI)-Induced Carcinogenesis

Recent studies have demonstrated that free radicals generated by Cr-mediated reactions can cause DNA strand breaks, base modification, lipid peroxidation, and nuclear transcription factor NF-κB activation, as discussed below.

1. *DNA strand breaks.* A significant amount DNA strand breaks occurred when DNA was incubated with Cr(VI) and ascorbate.[68] The addition of H_2O_2 enhanced the DNA damage. The amount of DNA strand breaks is associated with the amount of free radicals generated.
2. *8-OHdG formation.* The ·OH radical can interact with guanine residues at several positions to generate a range of products, of which the most studied one is 8-hydroxydeoxyguanosine (8-OHdG). The formation of this adduct is considered a marker to implicate free radical reactions in the mechanism of carcinogenicity of a variety of agents. Using high-performance liquid chromatography (HPLC) with electrochemical detection, it has been found that ·OH radicals generated by chromium-mediated reactions caused 2′deoxyguanosine (dG) hydroxylation to form 8-OhdG.[68,76]
3. *Lipid peroxidation.* Lipid peroxidation, an oxidative deterioration of polyunsaturated components of membrane lipids, is implicated in the etiology of many disease processes, including cancer. It has been reported that intraperitoneal injection of rats with Cr(VI) resulted in lipid peroxidation, which was demonstrated by an increase in thiobarbituric acid-reactive substance in liver and kidney.[77] Reaction of lipid hydroperoxides with chromium generate lipid hydroperoxide-derived free radicals.[78,79] These radicals can cause cell membrane damage, leading to increased intracellular levels of catalytically active iron, which, in turn, increases generation of ROS. These ROS may interact with chromatin and thereby act as tumor initiators and/or promoters.
4. *NF-κB activation.* It has been reported that ·OH radical functions as a message for NF-κB activation.[80] Because several chromium intermediates are able to generate ·OH radicals, it is likely that Cr(VI) is able to cause NF-κB activation. Indeed, Cr(VI) is capable of activating NF-κB in Jurkat cells.[81] The reduction of Cr(VI) to low oxidation states is required for Cr(VI)-induced NF-κB activation. Hydroxyl radicals generated by Cr(V)- and Cr(IV)-mediated Fenton-like reactions play a prominent role in the activation of NF-κB. It may be noted that NF-κB binding sites serve as enhancers for the c-*myc* oncogene, which is associated with the formation of Burkitt's lymphoma. It is possible that NF-κB activation and a subsequent expres-

sion of protooncogenes, such as c-*myc*, may play a role in the induction of neoplastic transformation by Cr(VI).

Conclusions

Carcinogenic particulates can act directly on the target cells or indirectly in combination with other co-carcinogens to exert an effect. Asbestos in this regard appears to act as a complete carcinogen for mesothelial cells, whereas it is more like a classic tumor promoter for bronchial epithelial cells. It is evident from the studies reported that carcinogens act via different mechanisms and that knowledge of such mechanisms may provide a basis for distinguishing the degrees of risk from various carcinogens. Asbestos and crystalline silica can promote cell injury, ROS generation, secretion of cytokines, and proinflammatory factors. These factors individually or in concert may promote cell differentiation and proliferation. In asbestosis and silicosis, there is a close relationship between fibrosis (asbestosis/silicosis) and lung cancer. It is likely that fibrogenesis may predispose the lung to the development of cancer in asbestosis and silicosis, because there are several mechanistic pathways common to both fibrogenesis and carcinogenesis.

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