Mechanisms of Carcinogenesis by Crystalline Silica in Relation to Oxygen Radicals

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The carcinogenic effects of crystalline silica in rat lungs have been extensively demonstrated in many experimental long-term studies using several animal species (20). These experiments have revealed that the inhalation of crystalline silica dusts can lead to the development of a variety of lung tumors, including alveolar type II cell tumors, mesotheliomas, and adenocarcinomas. The mechanisms by which crystalline silica induces these tumors are not fully understood, but it is believed that the inhalation of silica dusts results in the formation of reactive oxygen species (ROS), which can damage DNA and promote the development of tumors.

Introduction

In the past decade, the carcinogenic effects of crystalline silica in rat lungs have been extensively investigated using a variety of experimental models. These models include studies using Fischer 344 rats, Sprague-Dawley rats, and Wistar rats, as well as studies using hamsters. The results of these studies have shown that the inhalation of crystalline silica dusts results in the development of a variety of lung tumors, including alveolar type II cell tumors, mesotheliomas, and adenocarcinomas.

More recently, evidence has emerged suggesting that the inhalation of crystalline silica dusts can also lead to the development of an increased incidence of lung tumors in humans. This evidence comes from studies conducted in areas where crystalline silica is widely used, such as in the mining and manufacturing industries. The results of these studies suggest that the inhalation of crystalline silica dusts may be a significant risk factor for the development of lung cancer in humans.

Key words: crystalline silica, quartz, cristobalite, tridymite, silicosis, lung carcinogenesis, DNA binding, DNA damage, Janus Green B, neoplastic transformation, lung, alveolar type II cell, cytokines, TGF-β1

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point to a critical role for host factors. The susceptibility for fibrogenesis may not be determined by the same host factors that influence the carcinogenic response. We hypothesized (5,6,9) that the different pathways observed in three rodent species may correspond to differences in host susceptibility to fibrogenesis and to carcinogenesis in different subsets of the human population. Identification of the critical host factors in the three rodent species may lead to an understanding of susceptibility mechanisms in different groups of human subjects.

We decided to investigate the following problems: determination of reactive sites on crystalline silica surface; binding of crystalline silica surface to DNA and/or induction of DNA damage; the role of oxygen radicals in the DNA damage induced by crystalline silica; induction by crystalline silica of neoplastic transformation and/or chromosomal damage; influence of the granulomatous/fibrogenic lung reaction induced by crystalline silica (silicosis) on epithelial proliferation and carcinogenesis.

Detection of Reactive Sites on Crystalline Silica Surface

A newly developed spectrophotometric method measures surface adsorption of the cationic dye Janus Green B to crystalline silica particles in aqueous suspension (10). Twelve preparations of crystalline silica were assayed by this method and also for specific adsorption area by the Brunauer–Emmet–Teller (BET) method (11) of surface adsorption of nitrogen gas. Samples tested included 10 preparations of α-quartz: min-U-sil 5, five size-fractionated samples of min-U-sil 10, HF-etched min-U-sil 5, DQ-12, F600, and Chinese standard quartz. Two synthetic preparations of cristobalite and tridymite were also tested.

A strong linear correlation was found between the Janus Green B adsorption method and the BET method of measurement for all samples (r = 0.977). All crystalline silica samples tested, including the synthetic preparations cristobalite and tridymite, conformed to the same linear relationship. The correlation was strongest (r = 0.991) for the commonly derived size-fractionated min-U-sil samples (Figure 1). Among four standard α-quartz samples tested, min-U-sil 5 and F600 had the lowest specific surface areas, whereas DQ-12 and Chinese standard α-quartz had much higher surface areas. The linear relationship between Janus Green B binding and BET surface area suggests that the ratio of aqueous surface charge to surface area is relatively constant for different crystalline silica preparations.

Binding of Janus Green B to the negatively charged crystalline silica surface is consistent with a charge binding mechanism. Bound dye maintains its color, indicating that it remains in its ionic form. We are currently investigating the correlation of the binding of the cationic dye with surface charge, as determined by zeta potential, which measures mobility of suspended particles subjected to an electric field (12).

The polymer poly(2-vinylpyridine-N-oxide) (PVPNO) is believed to bind to silanol groups on the crystalline silica surface (13). We determined the binding of PVPNO to the same crystalline silica samples listed above, and also found it to be strongly correlated to the surface area as determined by the BET method (10). The binding of PVPNO to crystalline silica did not interfere with Janus Green B binding, indicating that they reacted at different ligand sites—silanol groups for PVPNO and ionized groups for Janus Green B, respectively (10).

The Janus Green B binding assay represents a useful new technique for the assessment of surface characteristics of crystalline silica samples. Its advantages, compared to the BET method, include improved sensitivity, lack of requirement for specialized instrumentation, and the ability to make rapid simultaneous determinations on multiple samples. Comparison of surface area and surface charge for different preparations of crystalline silica is important in understanding the relative activities of these preparations in studies on mechanisms of silicosis and silica-induced lung cancer.

Crystalline Silica Binding to DNA, DNA Damage and the Role of Oxygen Radicals

The possibility of an interaction between crystalline silica and DNA was investigated by Fourier transform infrared spectroscopy (FT-IR) (14). Upon coinoculation in aqueous buffer, alterations were observed in both DNA and quartz spectra, suggesting that a DNA–silica complex was formed as quartz interacted with DNA. DNA remained in the B-form conformation in the DNA–silica complex. Following coinoculation with quartz in H2O, the most prominent changes in the DNA spectrum occurred in the 1225 to 1000 cm⁻¹ region: the PO₂⁻ asymmetric stretch at 1225 cm⁻¹ was found to be increased in intensity and shifted to lower frequencies; the PO₂⁻ symmetric stretch at 1086 cm⁻¹ was markedly increased in intensity, whereas the band at 1053 cm⁻¹, representing either the phosphodiester or the C—O stretch of the DNA backbone, was significantly reduced in intensity (Figure 2).

When DNA was exposed to increasing concentrations of quartz in D₂O buffer, the DNA spectrum revealed a marked increase in intensity of the peak at 1086 cm⁻¹ and a progressive decrease in the intensity of the peak at 1053 cm⁻¹. Additional changes occurred in the area between 1600 and...
while linear with DNA: similar assay detected chelators (aqueous suspension showed modifications of alone, process, perturbation obtained techniques hydrogen bonding interaction between surface silanol groups and the phosphate–sugar backbone of DNA.

We suggest that the binding of quartz to DNA is important in the induction of DNA damage due to oxygen radicals. Others have shown that quartz particles in aqueous suspension produce oxygen free radicals, which are detectable by electron spin resonance (ESR) spin-trapping techniques (15,16). We recently reported that these quartz-derived radicals can cause damage to DNA in vitro, which can be monitored using a simple electrophoretic assay (17). Damage to linear DNA was detected as a smearing of discretely sized plasmid DNA bands following incubation with quartz, alone or in the presence of radical modifying agents (H2O2, SOD, catalase, deferoxamine, and other metal chelators) (17). Thus DNA serves as a sensitive indicator molecule for monitoring free radical production by quartz and the effects of in vitro manipulations of free radical pathways.

Production of oxygen free radicals by crystalline silica alone is a continuous process, as shown by the long incubation times necessary to detect DNA strand breakage by the electrophoretic assay. While linear DNA was stable in buffer alone, quartz produced increasing amounts of DNA damage over time, clearly detectable after an incubation period of more than 3 weeks. Five standard quartz preparations and two synthetic preparations of cristobalite and tridymite differed significantly in their ability to mediate in vitro DNA strand breakage (Figure 3).

The relative rates of DNA strand breakage by the tested preparations of crystalline silica were found to be directly correlated with the production of the oxidized DNA base, thymine glycol, as measured by gas chromatography–mass spectrometry (data not shown) (18).

The effects of modifying agents on DNA strand breakage are consistent with a mechanism of free radical production in which trace quantities of metal, adsorbed to the crystalline silica surface, are involved in catalyzing the production of hydroxyl radicals from H2O2 by the Haber–Weiss reaction. The H2O2 substrate is derived, at least in part, from the dismutation of superoxide, produced at the crystalline silica surface by the reduction of dissolved molecular oxygen (17).

We studied the effects of crystalline silica in cultured cell lines. Quartz particles, <5 μm (min-U-sil 5), were suspended in culture medium and added to cell lines of fetal rat lung epithelial cells (FRLE) or of mouse embryo fibroblastic cells (BALB/3T3/A51-1-1). Both cell lines actively internalized crystalline silica particles, mostly in cytoplasmic phagosomes. Electron microscopic observations showed that several quartz-treated cells contained small (<0.5 μm) particles of quartz in their nuclei, confirmed by energy dispersive X-ray spectroscopy (19,20). This finding suggests that a direct interaction of crystalline silica with the genetic material may occur in cells, following silica uptake.

The results so far obtained demonstrate the production of oxygen radicals by crystalline silica surfaces in aqueous buffer, and indicate that they mediate in vitro DNA strand breakage and formation of oxidized DNA bases. However, the hydroxyl radi-

![Figure 3. DNA damage by crystalline silica. Samples were incubated with λ Hind III digest DNA in 10 mM phosphate buffer, pH 7.4, at 37°C. One milligram of DNA was removed from the supernatant of a centrifuged reaction at specified time points and electrophoresed in 0.7% agarose (17). (A) DNA damage by crystalline silica in the presence of additional H2O2 (1.5%). At this time point (42 hr), DNA damage by CSOZ was not detectable (confirmed in repeated experiments), damage by tridymite was minimal, damage by HFMOZ was moderate, and damage by DQ-12 was extensive. DNA treated with other silica samples (MOZ, F600, and cristobalite) was completely degraded at 42 hr (not shown). Earlier time points showed damage to be most rapid at the cristobalite sample, followed by F600 and MOZ (not shown). (B) Acceleration of DNA damage by SOD. DNA damage by MOZ alone was detectable only after 4 weeks of incubation (data not shown). Manganese SOD (not shown) and Cu/Zn SOD both shortened the time to detectable DNA damage. The control dust, hematite (Fe2O3), did not cause DNA damage in the presence of SOD.](image)
cal, which is responsible for most if not all of this DNA damage, has a reaction distance of approximately 15 Å, less than the width of the DNA helix. Thus, in order for this radical to induce a mutagenic effect in vivo, silica particles and DNA would have to be very close to each other.

The results obtained by infrared spectrometry, showing that crystalline silica can bind to DNA at the phosphate backbone, suggest a likely mechanism for the effective induction of DNA damage. We propose that the DNA binding to the crystalline silica surface is important in silica carcinogenesis by anchoring DNA close to sites of oxygen radical production on the silica surface, so that the oxygen radicals are produced within a few Å from their target DNA nucleotides.

Neoplastic Transformation and Chromosomal Aberrations Induced by Crystalline Silica

The induction of cytotoxicity and neoplastic transformation by quartz was first reported in Syrian hamster embryo cells (21). We studied it in the BALB/3T3/A31-1-1 mouse embryo cell line, which had been previously characterized for transformation assays in our laboratory. Five samples of quartz (min-U-sil 5, HF-etched min-U-sil 5, DQ12, F600, and Chinese Standard Quartz) were tested at final quartz concentrations of 6.5, 12.5, 25, 50, or 100 μg/cm² (6,20). All tested quartz samples showed a dose-dependent induction of neoplastic transformation at lower doses, followed by a plateau response at higher doses. Morphologically transformed foci, subcultured and tested for tumorigenicity in nude mice, were all rapidly tumorigenic. Control and transformed cell lines were examined for karyotypes and chromosome abnormalities: all transformed cell lines showed one or more altered chromosomes not seen in the untreated cell line (6,20). These findings, to be reported in detail elsewhere, confirm at the cellular level that quartz consistently induced cellular lesions resulting in neoplastic transformation. Further studies are needed to investigate the mutagenic activity of crystalline silica in appropriate cellular systems, such as the human–hamster hybrid A cell line, in which a strong mutagenic activity was demonstrated for chrysotile and crocidolite asbestos (22). Additional studies, using cell line FRLE (23), are in progress in our laboratory (19), to investigate the effects of crystalline silica on this appropriate target cell type, which retains the characteristics of alveolar type II cells.

Mesenchymal/Epithelial Interactions and the Role of Cellular Mediators in the Pathogenesis of Silicosis and Associated Lung Carcinogenesis

We have considered the direct interactions of crystalline silica with target DNA and target cells in culture. The lung reactions to crystalline silica in vivo, resulting in silicosis and associated lung carcinogenesis, also are dependent on host factors, as demonstrated by the different response patterns in three rodent species, discussed above. Because in rats, the species most susceptible to both fibrogenesis and carcinogenesis, the induction of alveolar type II hyperplasia and lung tumors occurs in close association with silicotic lesions, we have hypothesized (6,9,24) that cellular mediators are important in the stimulation of the epithelial proliferative reaction. Such mediators may include various cytokines detected in the silicotic tissues (IL-1, IL-6, TNF-α, TGF-β), possibly mast cell products, as well as oxygen radicals produced by macrophages in the granulomatous reaction. Information on the role of these factors in the different animal models for crystalline silica is still scanty (6). Recent studies in our laboratory were devoted to the immunohistochemical localization of TGF-β1, a multifunctional growth factor, in quartz-induced lung lesions in rats (24). The results showed that TGF-β1 precursor (indicative of the site of synthesis) was localized in macrophages in the early stages of lung reaction to quartz, and more prominently after a few weeks from quartz exposure, in the hyperplastic alveolar type II cells. Mature TGF-β1 was localized, intracellularly, in macrophages and fibroblasts at the periphery of silicotic granulomas and in the stroma adjacent to hyperplastic alveolar type II cells, whereas the extracellular mature TGF-β1 was localized in the collagenous stroma adjacent to hyperplastic alveolar type II cells. Interestingly, the cells of adenomas retained a strong localization of TGF-β1 precursor, but those of carcinomas were negative, indicating that a downregulation of TGF-β1 occurs when these cells become malignant. Current studies on the localization of TGF-β1 in the lung of other quartz-treated species indicate that it is minimal in mice and not detected in hamsters, suggesting a critical role for TGF-β1 in the pathogenesis of silicosis-associated lung carcinogenesis. We are also investigating the expression and localization of proteins encoded by selected oncogenes and tumor suppressor genes, to identify the molecular pathways corresponding to this type of carcinogenesis.

Much further work is needed to characterize the critical host control mechanisms and pathways in animal models and in human subjects following exposure to crystalline silica.

Conclusions

The biologic effects of crystalline silica and other inhaled minerals, including neoplasia as well as fibrosis, research needs to be addressed to several issues, including the following: mechanisms by which DNA damage by mineral particles occurs in target cells; intracellular site(s) of such interaction, including the question of nuclear penetration of mineral particles and its significance; identification of the radical species generated and the conditions required for their generation and biologic activity in the cellular milieu; the role of scavengers and inhibitors in the pathways leading to specific biologic effects, in relation to cellular and tissue environments; possible differences in free radical mechanisms identifiable in animal species corresponding to different levels of susceptibility for fibrogenesis and carcinogenesis by crystalline silica.

The ultimate challenge is this area of research is to correlate the knowledge acquired through experimental studies—at the tissue, cellular, and molecular level—with the events taking place in the lungs of human subjects, in order to elucidate the mechanisms of lung injury, their inhibition, and their possible prevention.

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