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REACTIVE OXYGEN SPECIES AND SILICA-INDUCED CARCINOGENESIS

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Although silica has recently been designated as a carcinogen, its mechanism of carcinogenesis is not fully understood. Recent studies suggest that free-radical reactions may play an important role in the initiation and progression of cancer. This article summarizes literature on the generation of reactive oxygen species (ROS) directly from silica and from silica-stimulated cells. It also summarizes information concerning the role of ROS in silica-induced DNA damage as well as in silica-induced cell proliferation, including the effects of silica on the activation of nuclear transcription factors, induction of growth factors and oncogene expression, redox regulation of the p53 tumor suppressor gene, induction of apoptosis, and division of damaged cells. Understanding the role of ROS in silica-mediated reactions may help develop therapeutic agents to block silica-induced free radical reactions and thus prevent or attenuate silica-induced carcinogenesis.

Since antiquity silicosis has been known to occur in dusty work conditions. Other diseases contributed to by the inhalation of crystalline silica (silica) are bronchitis, tuberculosis, scleroderma, rheumatoid arthritis, renal disease, and emphysema. It is only in the past 20 years that a new hypothesis has been advanced that suggests that crystalline silica inhalation might lead to lung cancer. This was mostly based on experimental animal studies with limited information on humans. In 1986 an expert working group organized by the International Agency on Cancer (IARC) reviewed all the available studies and published a monograph stating that there was sufficient evidence for the carcinogenicity of silica in animals, while listing it as only a probable carcinogen to humans (IARC, 1986). Since the publication of that IARC monograph, much more information has emerged on crystalline silica and its carcinogenic potential in animals and humans (McDonald, 1996). In 1997, IARC published another monograph (IARC, 1997) concluding that there is now sufficient evidence in humans for the carcinogenicity of inhaled crystalline silica in the forms of quartz or crystobalite from occupational sources.

Although silica is now a documented carcinogen, the concentration-response relationship is still debated and many questions remain to be

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answered. For example, carcinogenesis induced by silica shows species differences. In mice, a single intratracheal instillation of silica gave rise to lung granulomas with minimal or moderate fibrosis, with neither persistent alveolar epithelial hyperplasia nor tumor induction; yet in rats treated in the same way, fibrosis, type II cell hyperplasia, and cancer were noted (Saffiotti et al., 1996). Comparable silica treatment in hamsters induced macrophagic granulomas without progressive fibrosis, no epithelial proliferation, and no tumors (Saffiotti et al., 1996). Mechanistic studies of silica-induced carcinogenesis are required to understand the role of host factors in these species differences. These studies are also necessary for the development of prevention and therapeutic strategies against silica-induced lung cancer.

Because silica is a newly established carcinogen, there are limited studies regarding its mechanism of action. Studies have demonstrated that freshly fractured silica particles generate silicon-based free radicals such as $Si^{\bullet}$, $SiO^{\bullet}$, and $SiOO^{\bullet}$ (Dalal et al., 1986; Fubini et al., 1987), and upon reaction with aqueous medium these particles generate H_2O_2, superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\bullet}OH$) (Shi et al., 1988, 1995; Vallyathan et al., 1988). Oxygen free radicals such as $O_2^{\bullet-}$ and $^{\bullet}OH$ and related oxygen reduction products such as H_2O_2 and $^1O_2$ are collectively called reactive oxygen species (ROS). Because ROS are known to be involved in the carcinogenicity of a variety of substances (Wiseman & Halliwell, 1996), we hypothesize that silica-mediated free-radical reactions may cause a persistent oxidative stress in the lung and play a key role in the mechanism of silica-induced carcinogenesis. This article reviews the evidence linking mostly our laboratory studies of free radical reactions to the mechanism of silica-induced carcinogenesis.

Current Mechanisms for Carcinogenesis

While the mechanisms of cancer development are complex, it is generally believed that four factors may be common to these processes.

1. Direct DNA damage. DNA strand breaks and hydroxylation of dG residues are typical examples. These types of DNA damage can lead to a series of mutations.
2. Excessive cell proliferation. Oncogenes, such as c-*jun*, c-*fos*, and c-*myc*, can lead to uncontrolled cell proliferation, a characteristic of cancer. Various cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α), are important mediators of growth and differentiation. Several transcription factors, especially nuclear factor κB (NF-κB) and activator protein 1 (AP-1), are involved in the expression of and response to growth factors and oncogenes.
3. Loss of growth regulation. Tumor suppressors function as physiological barriers against clonal expansion or genomic mutability and are able

to limit growth and metastasis of cells leading to uncontrolled proliferation. Mutated tumor suppressor genes can lose their tumor suppressor function resulting in the loss of growth regulation.

4. Division of damaged cells. ROS-induced lipid peroxidation products and a rise in intracellular level of Ca^{2+} can cause cell injury. If the injured cells are not removed, this injury can lead to uncontrolled division of damaged cells.

Because ROS are known to be involved in all these processes of carcinogenesis, it was hypothesized that silica-mediated free radical reactions cause persistent oxidative stress in the lung and lead to carcinogenesis (Figure 1). Data supporting this hypothesis are reviewed in this article.

ROS GENERATED DIRECTLY FROM SILICA

Silicon-Based Free Radicals

Dalal et al. (1986) and subsequently Fubini et al. (1987) have provided direct electron spin resonance (ESR) evidence for the generation of silicon-based free radicals from silica ground in air. A later study by Vallyathan et al. (1988) demonstrated that after 4 wk of storage of silica in air following grinding, only about 20% of the original ESR signal remains detectable. This observation indicates that the reactivity of freshly fractured silica may be different from that of aged dust. Measurements on silica-induced toxicity and lipid peroxidation indeed showed that freshly fractured silica is more toxic and induces a higher degree of lipid peroxidation than aged silica in vitro and in vivo (Shi et al., 1988; Vallyathan et al., 1988, 1992, 1995).

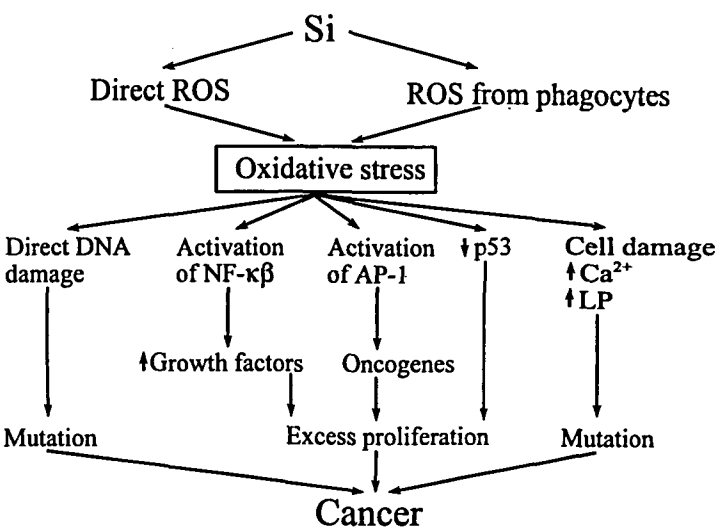

FIGURE 1. Schematic representation of silica-induced generation of ROS and possible role in carcinogenesis.

These studies suggest that free radicals generated from silica ground in air may be involved in the mechanism of silica-induced cellular injury.

H_2O_2

Marasas and Harington (1960) reported that silica particles could be employed as oxidants in a number of in vitro oxidations. They postulated that silica particles, upon reacting with water, could release certain oxidants that might have the potential to react with various biological constituents and thus cause tissue damage. Shi et al. (1988) have shown that freshly ground silica suspended in aqueous medium is able to generate H_2O_2 and that its yield, depending on the pH and the temperature of hydrolysis, is high enough to be measured by a standard analytical chemistry method, the $2MnO_4^-$ reaction ($2MnO_4^- + H_2O_2 + H^+ \rightarrow 5O_2 + 2Mn^{2+} + 8H_2O$). Subsequent studies of the mechanism involved in silica-induced $^{\bullet}OH$ generation confirm the formation of H_2O_2 from such a silica reaction.

$^{\bullet}OH$

Because the lifetimes of reactive free radicals are too short for them to be detectable directly by conventional ESR, a spin trapping method (Janzen & Blackburn, 1969) is frequently used. Using this method, Shi et al. (1988) and Vallyathan et al. (1988) have demonstrated that freshly fractured silica particles generate $^{\bullet}OH$ radical. The $^{\bullet}OH$ radical yield increases with prolonged grinding as the number of fracture planes increases. The yield decreases by more than half when these particles are stored in air for 4 d. $^{\bullet}OH$ radical scavengers, ethanol and dimethyl sulfoxide (DMSO), interact with $^{\bullet}OH$ radicals to generate carbon-centered radicals, derived from the reaction of $^{\bullet}OH$ radicals with ethanol or DMSO. These scavenging effects indicate that $^{\bullet}OH$ radicals are indeed generated.

To elucidate the mechanisms of $^{\bullet}OH$ radical generation, Dalal et al. (1990) have investigated the effect of the metal ion chelator deferoxamine. It was found that deferoxamine inhibits the $^{\bullet}OH$ yield, showing that deferoxamine may bind to the reactive center of the silica surface or to metal ions associated with the silica surface to block or attenuate the free radical generating capability of these particle. Catalase, a scavenger of H_2O_2, completely inhibits the generation of $^{\bullet}OH$, while addition of H_2O_2 enhances the yield. These results suggest that H_2O_2 is a key intermediate in $^{\bullet}OH$ radical generation. Both $Si^{\bullet}$ and $SiO^{\bullet}$ radicals might also react with H_2O_2 to produce $^{\bullet}OH$ radical and HO_2 ($O_2^{\bullet-}$) (Shi et al., 1989; Vallyathan et al., 1988). Since transition metal ions, such as Fe^{2+} and Fe^{3+}, are likely to be present as trace impurities in aqueous solution or on the silica surface, H_2O_2 could react with iron to generate $^{\bullet}OH$ radicals via Fenton chemistry.

$O_2^{\bullet-}$

ESR spin trapping measurements have shown that in the presence of a high concentration of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide

(DMPO), part of the oxygen radicals generated from silica particles can be trapped as $O_2^{\bullet-}$ (Shi et al., 1995). A high concentration of DMPO is required to trap $O_2^{\bullet-}$ due to the relatively low reactivity of $O_2^{\bullet-}$ toward DMPO. These studies have shown that $O_2^{\bullet-}$ is present on the surface of silica particles to react with metal ions or reactive centers and participate in chain reactions leading to free radical generation. $O_2^{\bullet-}$ may also be site-specifically generated and remain bound to the surface of silica particles. In an aqueous suspension of freshly fractured silica molecular oxygen is rapidly consumed, which appears to be the source of ROS generated by silica reactions (Shi et al., 1995).

$^1O_2$

ESR has been used to detect singlet oxygen ($^1O_2$) using sterically hindered 2,2,6,6-tetramethyl-4-piperidone to generate a stable free radical nitroxide. Utilizing this method, Shi et al. (1995) reported $^1O_2$ generation from an aqueous suspension of silica particles containing H_2O_2.

ROS RELEASE FROM SILICA-STIMULATED CELLS

In Vitro O_2 Consumption and ROS Release

Most of the knowledge of silica-induced lung cell injury comes from studies using phagocytes. The generation of ROS represents one of the main mechanisms by which phagocytes kill invading organisms. ROS production increases in response to stimulation and phagocytosis of microorganisms, particulates, and chemicals, resulting in a sudden increase in oxygen consumption called the "respiratory burst." Although ROS are produced at some level by all respiring cells, phagocytes are particularly effective in generating the entire spectrum of molecular oxygen-derived species (Freeman & Crapo, 1982). The activation of phagocytes can create oxidative stress in a tissue. Following the activation, much of the oxygen consumed by macrophages and neutrophils is directed toward the generation of $O_2^{\bullet-}$. Macrophages, monocytes, eosinophils, and neutrophils have been shown to release copious quantities of $O_2^{\bullet-}$, H_2O_2, and (in the case of neutrophils) HOCl following activation by either particulates or soluble stimulants, such as phorbol 12-myristate 13-acetate (PMA). $^{\bullet}OH$ can arise by secondary reactions such as that of HOCl with $O_2^{\bullet-}$ (Weiss, 1989) and that of H_2O_2 with transition metal ions in surrounding tissue ($HOCl + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + Cl^- + O_2$ and $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$) (Halliwell & Gutteridge, 1990).

Crystalline silica is a potent stimulant of the respiratory burst in alveolar macrophages (Castranova et al., 1990; Vallyathan et al., 1992). The respiratory burst (increase in oxygen consumption) is associated with an elevated production of ROS as demonstrated by the following observations: (1) Silica stimulates $O_2^{\bullet-}$ production. (2) H_2O_2 release is increased in alveolar macrophages exposed to silica. (3) Chemiluminescence and enhanced generation of ROS are increased after in vitro exposure of

macrophages to silica. In each of these three cases, the stimulatory effect of silica on respiratory burst activity is rapid, reaching a maximum within a few minutes of in vitro exposure. Using ESR spin trapping, Vallyathan et al. (1992) have shown that the phagocytosis of mineral dusts by rat alveolar macrophages and human neutrophils results in an enhancement of the generation of ROS at levels directly related to their known toxicity when exposure concentrations are normalized to an equal surface area of the dusts.

Silica-induced stimulation of ROS production by neutrophils has also been studied recently (Castranova et al., 1996). In vitro exposure of neutrophils to silica results in a drastic increase in chemiluminescence, an indication of ROS generation. Silica also stimulates the release of chemotactic agents, such as platelet-activating factor, macrophage inflammatory protein 2 (MIP-2), and cytokine-induced neutrophil chemoattractant (CINC), from alveolar macrophages (Kang et al., 1991; Driscoll, 1996; Driscoll et al., 1995). These factors would result in the infiltration of neutrophils into the air spaces, where the macrophage-derived platelet-activating factor and other related factors would stimulate oxygen metabolism in neutrophils.

In Vivo Silica-Induced ROS Release in Silicosis

Silica, compared to inert particles, causes greater damage to the blood–air barrier of the lung and a greater recruitment of polymorphonuclear leukocytes (Beck et al., 1982). Intratracheal instillation or inhalation of silica to rats potentiates the activity of pulmonary phagocytes; that is, oxygen metabolism (oxygen consumption, H_2O_2 release, and chemiluminescence) in response to an in vitro stimulant is augmented in pulmonary phagocytes harvested from silica-exposed animals (Castranova et al., 1996). Vallyathan et al. (1995) have recently evaluated the biochemical and pathologic changes in the lavagate and lungs of rats exposed to aged or freshly fractured silica. The results obtained show that exposure to freshly fractured silica, leads to enhanced generation of ROS, lung injury and inflammation. In human pulmonary phagocytes, elevated release of $O_2^{\bullet -}$ and H_2O_2 has been reported with chronic silicosis (Rom et al., 1987), while increased chemiluminescence has been reported with acute silicosis (Goodman et al., 1992; 1997).

Blackford et al. (1994) have shown that silica instilled into rat lung increases the mRNA for inducible nitric oxide synthase (iNOS) in alveolar macrophages. iNOS-dependent chemiluminescence was significantly increased after intratracheal instillation of silica compared with saline control group. As a free radical, NO may contribute to the lung's response to silica by inhibiting the mitochondrial respiratory cycle or DNA synthesis. It is also likely that NO reacts with $O_2^{\bullet -}$ produced by macrophages to form peroxynitrite ($ONOO^-$). The formation of $ONOO^-$ has been demonstrated in activated macrophages (Ischiropoulos et al., 1992) and endoethelial cells (Kooy & Royall, 1994). $ONOO^-$ is able to inhibit directly mitochondrial respiratory enzymes, reduce cellular oxygen consumption,

and inhibit sodium transport in membranes (Hu et al., 1994). $ONOO^-$ can also cause DNA strand breakage, activate poly(ADP)-ribose synthase (Sazabo et al., 1996), and deplete cellular enzymes, contributing to the overall mechanism of lung injury.

Free-Radical Generation from Silica-Induced Lipid Peroxidation

Lipid peroxidation, defined broadly as the oxidative deterioration of polyunsaturated components of membrane lipid, has been implicated in the pathogenesis of many degenerative disorders, including cancer (Yagi, 1982). Lipid hydroperoxides are produced from a variety of long-chain polyunsaturated fatty acid precursors via free-radical reactions involving molecular oxygen. The net result of these combined reactions is the generation of highly reactive and cytotoxic lipid radicals (e.g., LO• and LOO•). These radicals will generate new lipid hydroperoxides because of their close proximity to other lipids in biomembranes and will also damage membrane proteins. As discussed in the previous sections, freshly fractured silica particles generate •OH radical upon reaction with aqueous medium or H_2O_2. Thus, silica would cause lipid peroxidation via •OH-initiated reactions. A suspension of silica particles in contact with linoleic acid has indeed been reported to generate lipid-derived free radicals (Shi et al., 1994) and initiate lipid peroxidation (Vallyathan et al., 1988). The ability of silica particles to peroxidize lipids has been related to its potential for the generation of •OH; that is, the rates of both silica-induced lipid peroxidation and •OH generation decline markedly over the first 48 h after fracturing and remain relatively constant thereafter (Shi et al., 1988; Vallyathan et al., 1988). Free-radical scavengers, 1,3-dimethyl-2-thiourea (DMTU), dimethyl sulfoxide (DMSO), mannitol, and sodium benzoate, significantly inhibit silica-induced lipid peroxidation (Vallyathan et al., 1988). Catalase also exhibits an inhibitory effect. All these results point to the role of ROS in the mechanism of silica-induced lipid peroxidation.

In a study of workers exposed to silica or asbestos, Kamal et al. (1989) used plasma thiobarbuturic acid-reactive substances (TBARS) as an indicator of dust-induced lipid peroxidation products in vivo. Random samples of workers exposed to silica or asbestos were compared with healthy male controls. TBARS levels of exposed groups were significantly higher than those of controls. Neither age nor smoking was related to TBARS levels among either control or exposed workers.

ROLE OF ROS IN SILICA-INDUCED CARCINOGENESIS

DNA Damage

DNA Strand Breaks Using a DNA strand breakage assay, Daniel et al. (1992) and Shi et al. (1994, 1995) have shown that silica causes DNA strand breaks in vitro. Chemical etching of silica particles with hydro-

fluoric acid removes metal ion impurities and reactive centers created by fracturing and results in markedly diminished DNA damaging ability (Daniel et al., 1992). This DNA damage is blocked by catalase and by the •OH radical scavenging agents DMSO and sodium benzoate, indicating the role of •OH radicals (Daniel et al., 1992; Shi et al., 1994, 1995). The DNA strand breakage is dependent on the presence of molecular oxygen. Since in an argon environment little or no DNA damage is observed, ROS play a key role in silica-induced DNA damage. Although $O_2^{\bullet-}$ radicals may not be directly involved in silica-induced DNA damage, this radical may enhance the •OH radical generation via Haber–Weiss reactions. While these in vitro experimental conditions are not directly comparable to intracellular silica interactions, antioxidant defense, and DNA repair, the results do show that the ROS generated by silica reactions have the potential to cause DNA damage.

Infrared spectroscopy has been used to study the possible binding between DNA and the surface of silica (Mao et al., 1994; Saffiotti et al., 1994). The results show that silica causes modifications of the DNA spectrum, indicative of structural changes in the DNA phosphate backbone due to reorientation of the phosphate groups and their involvement in the DNA–silica interaction. Conversely, DNA–silica interaction was found to modify the silica spectra in the band corresponding to the SiOH group on the silica surface. The changes in the infrared spectra of silica and DNA following their interaction suggest that hydrogen bonds form between SiOH groups and the DNA phosphate backbone. The DNA binding to silanol groups on the surface of silica may provide a favorable environment for silica-induced DNA damage via free-radical reactions (Shi et al., 1989). Due to its very short life time, •OH radical can only react with targets located at sites close to its generation (Halliwell & Aruoma, 1991). The binding of silica to the DNA phosphate backbone provides a mechanism that anchors DNA strands close to the sites of radical production on the silica surface.

8-OHdG Formation •OH radical can interact with guanine residues at the C-8 position to generate a range of products, of which the most studied is 8-hydroxydeoxyguanine (8-OHdG) (Dizdaroglu, 1991). 8-OHdG is also generated by reaction of $^1O_2$ with guanine in DNA (Dizaroglu, 1991; Kohda et al., 1990; Shi & Mao, 1994). 8-OHdG is a conventional model to demonstrate ROS-induced DNA damage, because this modified base is important in mutagenesis and carcinogenesis (Floyd, 1987; Kasai & Nishimura, 1984). Its levels are directly correlated with carcinogenic effects in vivo (Floyd, 1987). Using high-performance liquid chromatography (HPLC) with electrochemical detection, it has been shown that silica particles in suspensions cause deoxyguanine (dG) hydroxylation to produce 8-OHdG (Shi, unpublished results). Catalase and sodium formate, a scavenger of •OH radicals, inhibit silica-induced 8-OHdG formation in vitro, demonstrating the role of ROS reactions in silica-induced 8-OHdG

formation. The $^1O_2$ generated by silica particles may also contribute to silica-induced 8-OHdG formation. However, there is little or no study regarding the relationship between silica-induced 8-OHdG generation and the development of silica-induced carcinogenesis.

Thymine Glycol Formation Pyrimidines in DNA can be attacked by •OH radical to give multiple products, such as thymine, glycols, and other end products (Dizdaroglu, 1991). Thymine can form cis and trans thymine glycols and other end products. Direct evidence of DNA oxidative damage by silica has been provided using a recently developed assay for production of thymine glycol (Daniel et al., 1995). Following co-incubation with DNA in aqueous suspension, silica produces thymine glycol, indicating that DNA strand breakage by silica in vitro is mediated by ROS. Thymine glycol may exhibit some mutagenic activity, and it can be lethal if not removed from the DNA before replication (Breimer, 1990).

DNA Damage Caused by Lipid Peroxidation Products Peroxidizing lipids produce a range of ROS including $^1O_2$, peroxyl radicals, and alkoxyl radicals. The radicals derived from lipid peroxidation have been demonstrated to cause the following types of damage to cellular systems (Vaca et al., 1988):

1. Cell membrane damage. This damage may lead to increased intracellular levels of catalytically active iron and thus increase generation of ROS (Halliwell & Aruoma, 1991). The latter may interact with DNA and thereby act as tumor initiators and/or promoters.
2. Site-specific cleavage of double-stranded DNA. It has been reported that incubation of DNA with oxidized linoleic acid induces DNA strand breaks. The addition of metal ions increases the formation of DNA strand breaks. The ability of metal ions to enhance this effect seems to correlate with the capacity of these ions to generate lipid-derived free radicals (Ueda et al., 1983).
3. DNA damage caused by the products generated from lipid peroxidation. These products include malondialdehyde and other groups of aldehyde products, such as hexanal and 4-hydroxynonenal (Vaca et al., 1988). Silica-generated lipid peroxidation products may cause cell injury through all of these processes.

Unregulated Cell Proliferation

Effect of Silica on Nuclear Transcription FκB The NF-κB protein is found in many different cell types and is a focal point for understanding how extracellular signals induce the expression of specific sets of early-response genes in higher eukaryotes, such as those regulating the secretion of growth promoters. In many cell types, ROS have been shown to activate the nuclear translocation of NF-κB by activating reactions leading to displacement of an inhibitor (IκB) from NF-κB in the cytoplasm (Baeuerle & Baltimore, 1996). Using the mouse macrophage cell line

RAW 264.7, Chen et al. (1995a, 1995b) have demonstrated that silica is a potent inducer of NF-κB activation. Chen et al. (unpublished results) have also studied the role of ROS in the mechanism of silica-induced NF-κB activation. Catalase blocks this silica-induced NF-κB activation, and H_2O_2 is involved in the activation of nuclear translocation of NF-κB in a range of cell lines (Schreck et al., 1991). SOD, which converts $O_2^{\bullet -}$ to H_2O_2, exhibits an opposite effect, that is, increasing silica-induced NF-κB activation in vitro. The metal ions of Fe(II) but not Fe(III) enhance the NF-κB activation, whereas the metal chelator deferoxamine reduces the NF-κB activation. Similar inhibitory effects were observed using ascorbate, formate, and the SiOH blocker poly(2-vinylpyridine *N*-oxide) (PVPNO) (Shi et al., 1989). These results indicated that silica-mediated $^{\bullet}OH$ radical generation via Fenton or Fenton-like reactions may be involved in the mechanism of silica-induced NF-κB activation.

Effect of Silica on Activator Protein 1 AP-1 is an important mediator of tumor promotion involved in a diversity of processes. This factor is a complex protein composed of homodimers and heterodimers of oncogene proteins of the Jun and Fos families. The genes encoding these proteins, c-*jun* and c-*fos*, are inducible by a variety of extracellular stimuli and function as intermediary transcriptional regulators in signal transduction processes leading to proliferation and transformation. The activity of AP-1 is modulated by several factors, including the redox state of the cell. Evidence suggesting the direct involvement of ROS in AP-1 activation has been obtained by using defined ROS-generating systems to challenge cultured cells. It appears that both H_2O_2 and $O_2^{\bullet -}$ are capable of inducing the expression of several early-response genes including c-*jun* and c-*fos*. While the detailed mechanism of ROS-mediated AP-1 activation is not well known, it has been suggested that AP-1 activation under oxidative conditions may be in part mediated by phosphorylation of Jun proteins (Sun & Oberley, 1996). Preliminary studies in our laboratories show that silica is also capable of inducing AP-1 activation, albeit at low levels. Further studies are under way to examine the role of ROS in silica-induced activation of this transcription factor.

Effect of Silica on Growth Factors The activation of alveolar macrophages results in the release of many cytokines capable of inducing fibroblast proliferation and collagen synthesis. Cytokines are implicated in the pathogenesis of chronic pulmonary fibrosis caused by inhalation of occupational dusts (Driscoll, 1996; Driscoll et al., 1996) and as a promoting factor for transformation initiated by certain chemicals in vitro (Komori et al., 1993). These macrophage-derived cytokines include interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), macrophage-derived growth factor (MDGF), and interferon γ. It has been documented that exposure of alveolar macrophages to silica can initiate excessive production of these cytokines and related inflammatory mediators caused by the

activation of several early response genes (Chen et al., 1994; Piquet et al., 1990; Zhang et al., 1993). At the molecular level, a common structural characteristic of these genes is the presence of one to five NF-κB binding sequences (κB) in the 5′-flanking region (Lowenstein et al., 1993; Tazawa et al., 1994). Thus, NF-κB is an important transcription factor regulating the expression of genes encoding those cytokines. Since silica is able to activate NF-κB, it may cause cytokine secretion via activation of this transcription factor.

Induction of Oncogene Expression Moderate concentrations of intracellular ROS influence gene expression as well as posttranslational modification of proteins. It has been reported that the redox state may modulate the formation of a functional Jun–Fos complex in vivo, controlling the oncogenic potential of AP-1 proteins. H_2O_2 and $O_2^{\bullet-}$ can induce c-*jun* and c-*fos* expression in a number of cell models, and overexpression of c-*jun* or AP-1 has been shown to cause cellular proliferation and transformation in those cells. For example, Janssen et al. (1994) reported that the induction of c-*jun* by asbestos in target cells of lung and pleura was associated with asbestos-induced carcinogenesis. Exposure of hamster tracheal epithelial (HTE) cells to H_2O_2 for 2 h causes a dramatic induction of c-*fos* and c-*jun*. Janssen et al. (1994) have also found that the carcinogenic potential of fibrous dusts correlated, in general, with their ability to induce proto-oncogenes. Although information on silica-induced oncogene expression is not yet reported in the literature, silica particles have the potential to cause expression of c-*fos* and c-*jun* because of their capacity to generate ROS. Redox state may also modulate the expression of another oncogene, c-*myc*, which plays a central role in the neoplastic transformation. It is known that c-*myc* is capable of transforming cultured cells and inducing tumors in transgenic animals. Since there is an NF-κB binding site associated with the c-*myc* gene, it is possible silica may regulate c-*myc* via ROS-induced activation of NF-κB. The mechanisms regulating the potential of silica-induced oncogene expression are currently under investigation in our laboratories.

Redox Regulation of the p53 Tumor Suppressor Gene p53 is a transcriptional activator that upregulates the expression of several genes controlling growth-inhibitory and apoptotic pathways. It is believed that p53 serves as a tumor suppressor by preventing the passage of genetic lesions in cells with DNA damage to a new generation of cells. It does this either by halting cell division to allow for DNA repair or by inducing apoptosis of damaged cells. Mutational inactivation of p53 has been found to be a frequent molecular alteration in human cancers, indicating the importance of the p53 in human carcinogenesis (Sun & Oberley, 1996). There are 10 cysteine residues in p53 protein. Redox regulation at a posttranslational level often occurs by reduction or oxidation of a disulfide bond. The reducing environment in cells is important for active p53 protein. Exposure of cells to silica particles may convert the cells to a pro-oxidant

state resulting from increased production of ROS or decreased expression of antioxidant enzymes. This oxidizing environment may render wild-type p53 conformationally mutant and give rise to the same biological outcome as p53 mutation. However, information is still not available regarding the effect of silica particles on p53 activity. This is an area deserving active investigation regarding the mechanisms of silica-induced carcinogenesis. ROS can also damage the p53 gene, and mutations in p53 in cancerous tumors are frequently of the type that could arise by ROS attack (Feig et al., 1994).

Induction of Apoptosis Apoptosis plays an important role in a variety of physiological conditions. Dysregulation of apoptosis may contribute to the pathogenesis of many diseases including cancer. In a recent study, Iyer et al. (1996) have shown that fibrogenic particulates, such as silica, caused apoptosis of human alveolar macrophages and suggested that the apoptotic potential of fibrogenic particulates may be important in silica-induced fibrosis and carcinogenesis. While further studies are needed to elucidate the mechanism, ROS have been implicated as potential modulators of apoptosis. There has been increasing evidence that ROS act as second messengers to mediate apoptosis and proliferation in response to a variety of stimuli. A link has recently been reported among apoptosis, p53, and ROS (Johnson et al., 1996). Recent studies (Van Antwerp et al., 1996; Wang et al., 1996) have shown that the activation of NF-κB suppresses the signal for cell death and protects cells from programmed death. Since silica is able to generate ROS and activate NF-κB, it is likely that silica may alter p53 gene expression. Investigation of the relationship between silica-mediated free-radical reactions, NF-κB activation, alteration in p53 gene expression, and apoptosis should improve our understanding of the mechanism of silica-induced carcinogenesis.

Division of Damaged Cells As stated earlier, silica is able to induce lipid peroxidation, which is a multistep free-radical-mediated reaction. The intermediates and products resulting from their reactions can cause DNA damage and cell injury. Another mechanism of silica-induced cell injury is through an increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$). A disruption of intracellular Ca^{2+} ($[Ca^{2+}]_i$) homeostasis may exacerbate free radical reactions, activate endonuclease, and alter signal transduction pathways. An increase in free cytoplasmic Ca^{2+} is frequently associated with early development of cell injury (Nicotera et al., 1990; Farber, 1990). Exposure of cells to reactive peroxides and several chemicals has been shown to induce a rise in $[Ca^{2+}]_i$ and subsequently cause cell death in a wide variety of cell systems, including cardiac myocytes (Josephson et al., 1991), hepatocytes (Masaki et al., 1989) and alveolar macrophages (Forman et al., 1987). Antioxidants such as α-tocopherol and the calcium chelator ethylenediamine tetraacetic acid (EDTA) protect the cells from injury (Reed, 1990). Exposure of phagocytes to ROS-generating particles such as silica has been shown to induce a rise in $[Ca^{2+}]_i$ in a dose-dependent man-

ner (Rojanasakul et al., 1996). In cultured rat alveolar macrophages, silica significantly increases $[Ca^{2+}]_i$ and causes cell injury.

CONCLUSIONS

The fracturing of crystalline silica generates silicon-based free radicals. Upon reaction with water or H_2O_2, silica is able to generate ROS. ROS can also be generated by silica-stimulated phagocytic cells. It is hypothesized that silica-mediated free radical reactions cause persistent oxidative stress in the lung leading to cancer (Figure 1). ROS and silica-generated lipid peroxidation products can directly damage DNA via strand breaks and formation of such modified bases as 8-OHdG and thymine glycol, which may lead to mutation. Through ROS, silica is also able to activate nuclear transcription factors, enhance secretion of growth factors, induce oncogene expression, and cause mutation of tumor suppressor genes, leading to division of damaged cells and carcinogenesis. Biological systems are normally protected against oxidative injury by enzymic and nonenzymic antioxidants. When the balance between pro-oxidants and antioxidants shifts in favor of pro-oxidants, silica-induced cellular damage via oxidative stress occurs. The difference in intra- and extracellular antioxidant levels among different species such as mice and hamsters may explain the different susceptibility to silica-induced carcinogenesis. Development of therapeutic agents to enhance intra- and extracellular antioxidant levels and to block ROS may mitigate this processes and thus prevent or attenuate silica-induced carcinogenesis.

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