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The Role of p53 in Silica-Induced Cellular and Molecular Responses Associated with Carcinogenesis

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Crystalline silica (silica), a suspected human carcinogen, produces an increase in reactive oxygen species (ROS) when fractured using mechanical tools used in several occupations. Although ROS has been linked to apoptosis, DNA damage, and carcinogenesis, the role of enhanced ROS production by silica in silica-induced carcinogenesis is not completely understood. The goal of this study was to compare freshly fractured and aged silica-induced molecular alterations in human immortalized/transformed bronchial epithelial cells (BEAS-IIB) and lung cancer cells with altered (H460) or deficient (H1299) p53 expression. Exposure to freshly fractured or aged silica produced divergent cellular responses in certain downstream cellular events, including ROS production, apoptosis, cell cycle and chromosomal changes, and gene expression. ROS production increased significantly following exposure to freshly fractured silica compared to aged silica in BEAS-IIB and H460 cells. Apoptosis showed a comparable enhanced level of induction with freshly fractured or aged silica in both cancer lines with p53 functional changes. p53 protein was present in the BEAS-IIB and was absent in cancer cell lines after silica exposure. Exposure to freshly fractured silica also resulted in a rise in aneuploidy in cancer cells with a significantly greater increase in p53-deficient cells. Cytogenetic analysis demonstrated increased metaphase spreads, chromosome breakage, rearrangements, and endoreduplication in both cancer cells. These results suggest that altered and deficient p53 affects the cellular response to freshly fractured silica exposure, and thereby enhances susceptibility and augments cell proliferation and lung cancer development.

In 1997, the International Agency for Research on Cancer (IARC) classified crystalline silica (silica) as a Group I human carcinogen (IARC, 1997). However, the interrelationships between silica, silicosis, and lung cancer have been criticized and debated based on the lack of consistency in animal and human studies. Studies performed in animals showed only strong evidence in rats, with no tumors seen in mice, hamsters, guinea pigs, or rabbits (ILSI, 2000; Saffiotti et al., 1996). The (1) inconsistencies in animal species, (2) insufficient replication of evidence in epidemiologic studies, and (3) lack of dose response suggest that there is no causal relationship between silica exposure and cancer (Hessel at al., 2000). Therefore the decision by IARC raised a debate among scientists and brought into question the basis and validity of many inconsistent results in epidemiological studies. Although several studies did not find an association between silica and lung cancer without silicosis, there was some evidence suggesting that the presence of lung cancer may provide increased silicosis detection (Checkoway et al., 1999). The absence of evidence between silica exposure and lung cancer may also likely be due to the limitations in the detection of lung cancer in early stages of carcinogenesis. This uncertainty is further confounded by the lack of mechanistic or molecular evidence of support in animal and cellular studies leading to silica-induced lung cancer development.

Strong evidence exists showing that fracturing silica results in the generation of increased levels of reactive oxygen species (ROS) on cleavage planes, and these species react with water to generate hydroxyl (•OH) radicals (Vallyathan et al., 1988; Shi et al., 1988). Freshly fractured silica as produced in occupational workplaces, such as by sandblasting, rock drilling, tunneling, and other operations, is biologically more reactive and produces enhanced lung injury and disease (Vallyathan et al., 1988; Shi et al., 1988). At present it is unclear how this increased production of ROS results in activation of various signaling pathways activation that has been linked to signal transduction and gene expression involved in cell proliferation, apoptosis, and carcinogenesis. Several in vitro and in vivo studies showed that many intracellular activations and regulation of molecular events are directly or indirectly regulated, or influenced by redox status (Allen & Tresssini, 2000; Shi et al.,

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1998, Castrunova, 2004). Elucidation of the molecular events and the signaling pathways directly regulated by ROS that are associated with carcinogenesis may provide a rationale for developing strategies for prevention or amelioration of disease. Furthermore, elucidation of mechanistic events associated with the development of lung cancer may help to refine and support epidemiologic studies.

In addition to the ROS-induced enhanced activation of molecular mechanisms in the genesis of lung cancer, development of cancer following exposure to silica in some cases may be a result of interindividual variations in activation of certain signaling pathways. This phenomenon, referred to as the "two-hit hypothesis" (Knudson, 1996), relates to host susceptibility in the multistage development of cancer. When a normal cell encounters a damage hit at the molecular level to the DNA, the cell may die by apoptosis if the damage is extensive, or may succeed in repairing the damage to DNA. If a second hit occurs to the same damaged DNA, this may lead to carcinogenesis.

One main component of signal transduction response in carcinogenesis is the transcription factor p53, which regulates the expression of genes involved in carcinogenesis. The activation of the p53-regulated genes is the main cell cycle checkpoint control, responsible for determining whether or not damaged DNA moves forward to DNA repair, or is disposed off via cellular apoptosis. Mutations in the p53 genes or its regulatory family members are among the most common genetic alterations in human malignancies (Greenbalt et al., 1994). Additional support for this hypothesis was the demonstration that freshly fractured silica produced direct DNA damage, including binding with deoxyribonucleic acid and molecular mechanistic changes inducing lung injury (Vallyathan et al., 1998; Daniel et al., 1995; Shi et al., 1994, 2001). Therefore, the purpose of the present study was to investigate whether the genetic variations in signal transduction pathways activated by freshly fractured silica exposure altered the cellular responses to normal and cancer cells with different p53 expression. Given the role of p53 alterations in the majority of cancers, the role of p53 was investigated in response to ROS production, apoptosis, and cell cycle control following exposure to both freshly fractured and aged silica. To meet these goals, the effects of both freshly fractured and aged silica were examined on three human pulmonary cell lines: a normal bronchial epithelial line (BEAS-IIB), a human adenocarcinoma cell line with altered p53 expression (H460), and a non-small-cell lung cancer cell line deficient in p53 (H1299). Measurements of ROS production, cell cycle parameters, and apoptosis were analyzed for all three cell lines exposed to freshly fractured or aged silica. Differences in responses to freshly fractured or aged silica producing high and low levels of ROS and the related p53 expression and subsequent changes in molecular events in these cell lines may help to relate the role of p53 in response to silica exposure and lung cancer development.

MATERIALS AND METHODS

Cell Culture

Analysis was performed in three cell lines: normal human bronchial epithelial cells (BEAS-IIB) grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS); human adenocarcinoma cells (H460) grown and maintained in RPMI 1640 with 2 m*M* glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 m*M* HEPES, 1 m*M* sodium pyruvate, and 10% FBS at pH 7.4; and human non-small-cell lung cancer cells (p53 –/-–cells, H1299) also grown and maintained in RPMI 1640 with 2 m*M* glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 m*M* HEPES, 1 m*M* sodium pyruvate, and 10% FBS at pH 7.4. All cells were maintained at 37°C in 5% CO_2. Cells were removed with 0.25% trypsin, 0.53 m*M* ethylenediamine tetraacetic acid (EDTA) solution and left at room temperature until detached.

Silica Exposure

Freshly fractured silica was prepared according a method described elsewhere and used within 30 min for cell exposure (Ding et al., 1999). Stock Min-U-Sil (U.S. Silica Co., Berkeley Springs, WV) was ground for 30 min using an agate ball mill (IEC Centrifugal Ball Mill, Fritsch, Germany), as described earlier. To obtain respirable size particles, the fractured silica was sieved through nylon mesh filters using a shaker for 10 min. Purity was checked using x-ray spectrometric analysis, and size was checked using scanning electron microscopic analysis, which indicated that the ground silica had a mean diameter of 3.7 μm and purity of 99.5%. The sieved silica was prepared in normal saline and used for the exposure of cells at a final concentration of 500 μg/ml for all studies except for electron spin resonance (ESR) measurements, for which 1 mg/ml was used. Cells were counted and seeded in either 1×10^6 cells/flask with regular media (enzyme analysis) or 2.5×10^5 cells/well (enzyme-linked immunosorbent assay [ELISA], DNA and RNA isolation) in starving medium (0.5% FBS) for 24 h. All cells were washed with PBS to be free of culture medium before exposure to silica. Cells for ESR analysis were fed as usual and resuspended at a concentration of 2×10^6 cells/ml in PBS pretreated with Chelex-100 (Sigma Chemical Co., St. Louis, MO). All the aged silica studies were conducted using samples from the corresponding freshly fractured silica aged for 1 wk in closed glass containers. All optimal exposure parameters were determined following time- and dose-response analysis (data not shown). All exposures were performed at least in triplicate samples of freshly fractured or aged silica prepared in different days.

Western Blot Analysis

All chemicals for Western blot analysis were obtained from Sigma Chemical Co., St. Louis, MO. Protein was extracted and isolated from the silica-treated cells and medium of treated cell

lines 24 h after exposure using 2% sodium dodecyl sulfate (SDS), in 50 m*M* Tris-HCl buffer, pH 7.4, containing 150 m*M* NaCl, 1.5 m*M* $MgCl_2$, 10% glycerol, 1% Triton X-100, 1% aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) protease inhibitors following procedures described previously (Sargent et al., 1996). Thirty micrograms of protein was resolved on 10% precast polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). The PVDF membranes were incubated with mouse anti-p53 1/200 (Santa Cruz sc-126). The blots were then developed with CDP-Star according to the manufacturer's instructions (New England Biolabs, Beverly, MA) and exposed to x-ray film. The membranes were then stripped in guanidine hydrochloride buffer, pH 7.4, and re-probed with human glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH 1/200) (Ambion, Austin, TX). Densitometry was performed and normalized to each sample's GAPDH (data not shown).

Apoptosis

Apoptosis was measured by a cell death detection ELISA kit (Roche Diagnostic, Montclair, NJ). This assay is based on a "sandwich enzyme immunoassay" using mouse monoclonal antibodies directed against DNA and histones according to the manufacturer's specifications (Roche Diagnostic, Montclair, NJ). The cell death detection assay measures the amount of histone bound to DNA in each sample involved in apoptosis. The samples are placed in streptavidin-coated plates and incubated in a mixture with antihistone–biotin and anti-DNA-peroxidase. The histone component of nucleosome is captured by anti-histone–biotin antibody and anti-DNA-peroxidase binds to nucleosomes. The unbound antibodies are removed and the immunocomplex is treated with ABTS substrate, and absorbance is measured photometrically at a wavelength of 405 nm using substrate solution as a blank. A DNA–histone complex serves as a positive control, and results are expressed as relative differences in absorbance caused by histone-associated DNA fragments of nucleosomes of apoptotic cells. The results are presented as percentages compared to the positive control producing 100% total apoptosis.

Electron Spin Resonance

Electron spin resonance (ESR) measurements were carried out using a Bruker ESR 300E spectrometer and a flat cell assembly. Hyperfine splittings were measured (0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. Cells (1×10^6) were incubated in a medium containing 0.5 ml PBS, 200 m*M* 5,5′-dimethyl-1pyrroline 1-oxide (DMPO), and 1 mg freshly fractured or aged silica for 3 min. After the exposure the samples were filtered through a 0.45-μm filter and transferred directly to flat ESR cells and read within 5 min. The ESR settings and experimental conditions were similar to a standardized protocol used for all cellular studies as described previously (Vallyathan et al., 1995).

Flow Cytometry

Cells were treated as already described, trypsinized from flasks following 24 h of exposure, and fixed in 70% ethanol for 1 h at 4°C. Following fixation, cells were pelleted and resuspended in PI/RNase stain buffer (BD PharMingen, San Diego, CA) for 15 min at room temperature. Cells were then analyzed with a FACscalibur (BD) and data were retained in CellQuest (Becton Dickinson, Franklin Lake, NJ). Cell cycle analysis was performed with ModFit LT software.

Cytogenetic Analysis

When cells were 70% confluent, 1 mg/ml colcemid (Invitrogen, Gaithersburg, MD) was added to block the cells in metaphase. After 10 h cells were incubated with 0.075 *M* KCl (Sigma, St. Louis, MO) for 30–90 min and then fixed using 3:1 (v/v) methanol/acetic acid (Sigma, St. Louis, MO**)**. Harvested metaphase preparations were dropped on slides and stained with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). At least 50 metaphase spreads of good quality were photographed and analyzed for breakage and gross chromosome aberrations using a Zeiss Axiophot microscope and Applied Imaging Genus software. Breakage was defined as a break across the entire chromosome arm. Dicentrics having two centromeres were noted. Gross translocations were identified based on size and centromere location as well as DAPI banding pattern.

Gene Expression

RNA was isolated as previously described (Gwinn et al., 2005). Briefly, 700 μl TRIzol was added to each well and left at room temperature for 5–10 min. The slurry was removed and passed through a pipette multiple times before placing in a 1.5-ml microfuge tube containing 100 μl chloroform. The tubes were capped, shaken for 10 s, and let stand at room temperature for 5–10 min. Samples were then centrifuged for 10 min at 12,000 × g at 4°C and the organic layer was removed (~200 μl) and placed in a 2-ml screw-cap tube containing 500 μl isopropanol. All samples were incubated overnight for 12 h at −20°C to precipitate and then centrifuged for 30 min at 12,000 × g at 4°C. The isopropanol was decanted, and samples were washed twice with 80% EtOH in diethyl pyrocarbonae (DEPC)-treated water. Samples were air-dried and resuspended in 20–30 μl of DEPC-treated water. Concentration of RNA was determined by spectrophotometry and samples with A_{260}/A_{280} of 1.8–2.1 were used for microarray analysis. The RNA samples were processed as per manufacturer's instructions and analyzed on the Superarray DNA damage signaling pathway microarray product number OHS-029 (Superarray,

Gaithersburg, MD). The same RNA was also used to confirm data by reverse-transcription polymerase chain reaction (RT-PCR). RT-PCR was performed following guidelines supplied with the RT^2 Real-Time and End-Point RT-PCR assay (Superarray). Briefly, 0.5 µg RNA was mixed with Buffer P and incubated for 70°C for 3 min. To this mix, the RT-PCR cocktail (buffer, reverse transcriptase, RNase inhibitor) was added and incubated for 60 min at 37°C. This reaction was then stopped by incubation at 95°C for 5 min. The mixture was then added to the PCR cocktail mix containing master mix and selected primers. A two-step PCR was then performed in cycles at 95°C/10 min; 95°C/15 s/40 cycles; and at 60°C/1 min. At the completion of cycles data was analyzed using the $\Delta\Delta C_T$ method.

Statistical Analysis

Data were analyzed using the SAS/STAT software, Version 9.1 of the SAS System for Windows. Dependent measures were analyzed using a two-way (treatment by cell strain) analysis of variance. Pairwise differences were evaluated using Fisher's least significant difference test. Data from the Superarray microarrays were analyzed using software provided by the company. Cytogenetic data was analyzed using chi-square statistics. The criteria for significance were set at $p < .05$.

RESULTS

Electron Spin Resonance

Figure 1 shows the typical 1:2:2:1 characteristic spectral pattern of the DMPO-OH adduct produced in the presence of DMPO following exposure to both freshly fractured and aged silica. Differences were found between treatments and between cell lines. Freshly fractured silica elicited a greater response than aged silica in all cell lines (Figure 1). The p53-deficient and p53-altered cancer cell lines showed a significantly increased •OH radical production following exposure to both fresh and aged silica exposure compared to wild-type BEAS-IIB cells. The wild-type BEAS-IIB cells and the H460 cells showed a significant increase in •OH production following exposure to freshly fractured silica as compared to aged silica exposure in the same cell lines. The p53-altered H1299 cells, on the other hand, showed no significant differences between fresh and aged silica exposures. There were no significant differences between freshly fractured silica exposure in all three cell lines; however, there was a significant difference related to genotype and •OH production levels following exposure to aged silica (Figure 1).

Apoptosis

Results of the cell death detection ELISA assay performed on all cell lines exposed to freshly fractured or aged silica are presented as percent apoptosis as compared to control cells (Figure 2). BEAS-IIB wild-type cells showed minimal apoptosis using this early biochemical marker of apoptosis measure, and apoptosis was also not significant with aged silica. In H460 adenocarcinoma cells with altered p53, significantly increased apoptosis was found in cells exposed to both freshly fractured and aged silica as compared to BEAS-IIB and H1299 cells, although no significant change was seen between the two treatments. Apoptosis also increased significantly in H1299 non-small-cell lung cancer cells exposed to aged silica, compared to freshly fractured silica. Statistical analysis between cell types following all treatments showed a significant difference in apoptosis, although the only significantly increased apoptosis with both fresh and aged silica exposures as compared to the control was in p53-altered H460 cells.

Western Blot Analysis

Of the three cell lines analyzed, only the wild-type cell line showed any significant p53 protein expression, with and

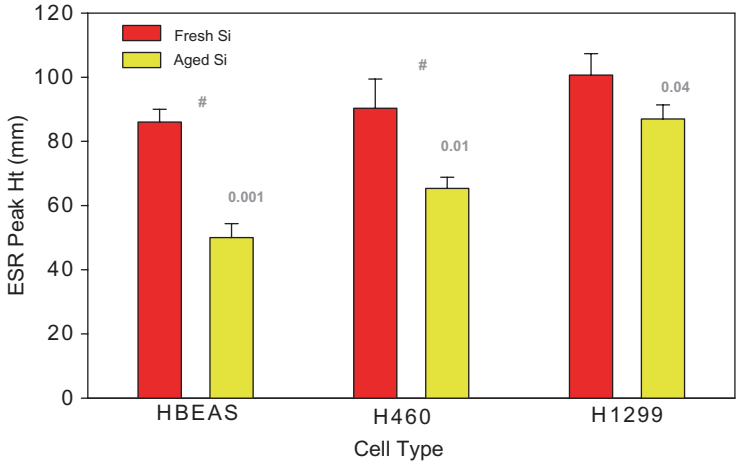

FIG. 1. Electron spin resonance (ESR) studies showing the differences in •OH radical generation by the normal and two cancer cells exposed to freshly fractured or aged silica. Exposure to (1 mg silica) freshly fractured silica generated more •OH radicals from all the cell lines compared to aged silica.

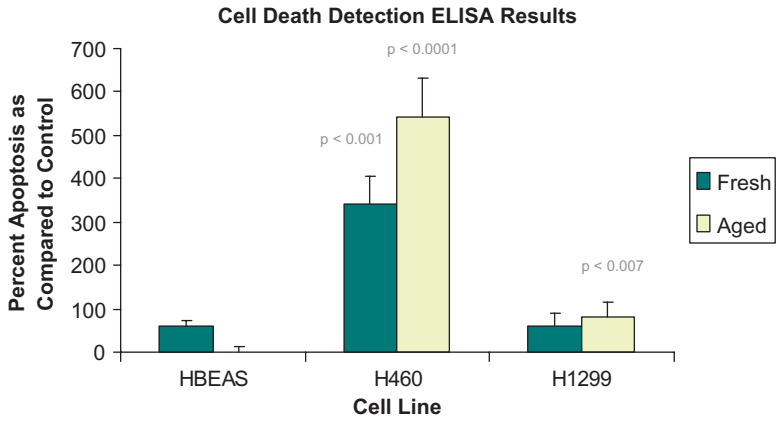

FIG. 2. Apoptosis measured by the cell death detection ELISA in the normal and two cancer cell lines is presented as percentage compared to controls for each cell type.

without silica exposure. However, although the H460 cell lines are described in the literature as p53 wild-type, no study was found in the literature to specifically address the levels of p53 expression in H460 cell line. From the results presented here, it is apparent that p53 protein levels in H460 as well as H1299 are similar or missing in response to silica exposure (Figure 3).

Flow Cytometry

In BEAS-IIB cells exposed to freshly fractured or aged silica showed no change in aneuploidy after 6 and 24 h. In contrast, both cancer cell lines exposed to freshly fractured silica as compared to those exposed to silica aged for 1 wk showed significant increases in aneuploidy after 6 and 24 h of exposure. In the H460 cell line, aneuploidy was 53.46% and 48.315% after 6 and 24 h of silica exposure, respectively (Figure 4a). The greatest marked increase in aneuploidy was seen in the H1299 cell line with freshly fractured silica exposure, after 6 h showing an increase of 98.79% and after 24 h an increase of 61.95%. The wild-type BEAS-IIB cells showed no significant change in any cell cycle parameters following either fresh or aged silica exposures, and both the cancer cell lines regardless of p53 expression showed a significant rise in cells in diploid S phase following exposure to freshly fractured silica after 6 h (Figure 4 b).

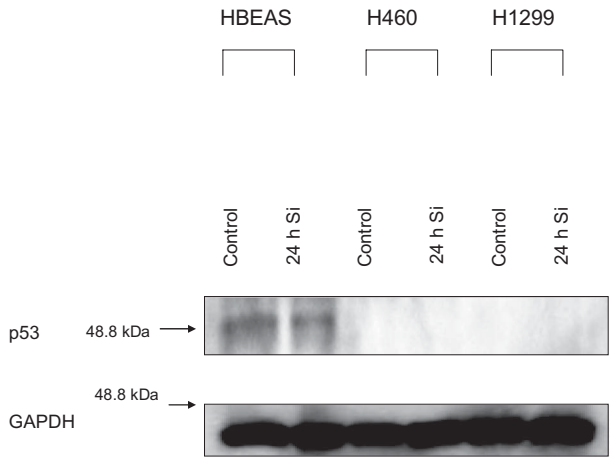

FIG. 3. Levels of p53 protein expression as monitored by Western blot analysis in normal and two cancer cell lines following exposure to freshly fractured silica.

Cytogenetic Analysis

Results of cytogenetic analysis demonstrated chromosome breakage and translocations in both cancer cell lines (Table 1 and Figure 5a). Although the breakage was increased in immortalazed normal BEAS-IIB cells after 6 and 24 h of silica exposure, the breakage was not statistically significant. The H460 cells showed dicentric chromosomes as early as 6 h. Dicentric chromosomes were also observed in the normal cells, the H1299 cells, and to a greater extent in the H460 adenocarcinoma cells at 24 h (Figure 5b). Furthermore, this analysis showed a marker chromosome in the H460 adenocarcinoma cells following exposure to freshly fractured silica (Figure 5b). In addition, this cell line also displayed an increased percentage of endoreduplication (2/50) following exposure to silica (Table 1). Neither the normal cell line nor the p53-deficient cell line showed these types of damage following exposure, though all three cell lines demonstrated some amount of chromosome breakage, with the greatest amount in the H460 cell line (Table 1 and Figure 5a). These results suggest that exposure to freshly fractured silica for 6 h results in DNA damage that has begun to be repaired by 24 h of exposure time in wild-type BEAS-IIB cells.

Gene Expression

Differences in gene expression of DNA damage signaling pathway genes were found between cell strains, with increased variation following exposure to freshly fractured silica. Among the 104 genes on the array used for analysis, the following 7 genes showed variation potentially related to differences in p53 expression in the 3 cell lines: p53 binding protein (p53BP1),

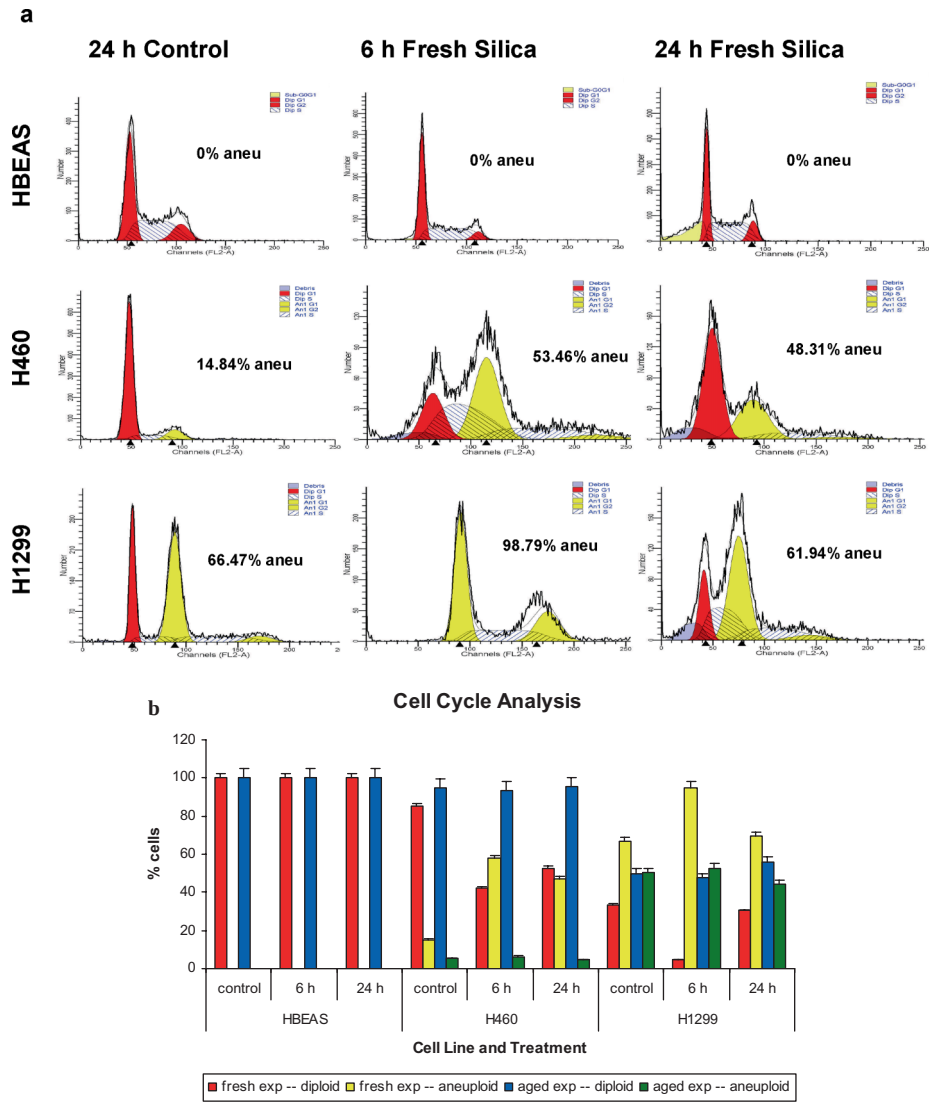

FIG. 4. Results of flow cytometric analysis of normal and two cancer cell lines exposed to freshly fractured or aged silica. The normal cells showed no increase in aneuploidy, while both the cancer cell lines displayed a significant increase in aneuploidy.

ATM, MDM2, BCL6, PCNA, ERCC3, and RAD23. Differences in gene expression as compared to untreated controls in the same cells showed significant severalfold increases in some of the these genes, ranging from 2.12- to 708.97-fold (Table 2). Select genes were further analyzed by RT-PCR to confirm the microarray results. RT-PCR results varied somewhat from those found on the array analysis. However, for the seven genes listed, RT-PCR results confirmed parallel alterations in gene expression and validated gene array analysis. All the 104 genes that showed upregulation by gene array were not analyzed by RT-PCR.

DISCUSSION

Gene–environment interactions have been implicated in the genesis of many diseases. This study was designed to determine whether genetic alterations to the p53 gene might alter the response to silica containing different levels of ROS. The p53 gene is commonly mutated in human cancers (Greenblatt et al., 1994) and is involved in control of DNA damage repair (Pfeifer et al., 2002), apoptosis (Xiao et al., 2000), and cell cycle progression (Robles et al., 2002). In response to DNA damage the p53 protein suppresses carcinogenesis by initiating cellular responses such as inhibition of cell cycle, and apoptosis. Therefore, the goal of this investigation was to determine whether there was a variable response in molecular events in cells with differential p53 expression following exposure to freshly fractured or aged silica.

Increased ROS production was shown in a variety of cell types following exposure to silica (Kang et al., 2000; Zeidler et al., 2003; Castranova, 2004; Shi et al., 1998, 2001). Several critical signaling events are linked to ROS generation, including production of inflammatory cytokines, chemokines,

TABLE 1
Cytogenetic Analysis of Chromosome Breakage Produced by Exposure to Freshly Fractured Silicas

Cell Strain &Treatment	Breaks	Dicentric	Marker Chromosome	Other
HBEAS				
Solvent	2.0% (1/50)			
6 hour	10.4% (12/115)			
24 hour	8.0% (4/50)	2.0% (1/50)		
H460				
Solvent	8.0% (4/50)			
6 hour	44.0% (22/50)	8.0% (4/50)		
24 hour	30.0% (15/50)	8.0% (4/50)	10% (5/50)	Endoreduplication Exchange
H1299				
Solvent	8.0% (4/50)			
6 hour	16.0% (8/50)			
24 hour	34.0% (17/50)	4.0% (2/50)		

Note. The two tumor cells lines H460 and H1299 demonstrated increased breakage at 6 and 24 h after silica exposure.

proliferative factors that are critical to the fibroblast activation, and initiation of cellular hyperplasia (Castranova, 2004). The results presented in Figure 1 show a pattern of ROS production being increased in response to freshly fractured or aged silica exposure. Exposure to aged silica was shown previously to result in less ROS production as compared to freshly fractured silica (Vallyathan et al., 1998; Ding et al., 1999, 2001). This is consistent with the results described here for all three cell lines in response to aged silica; however, both cancer cell lines show a moderate increase in ROS production when exposed to fresh or aged silica compared to the normal cells. There were no significant differences between levels of ROS production in all three cell lines following exposure to freshly fractured silica. These results do suggest, however, that the presence of a functional wild-type p53 does hinder ROS production when cells are exposed to aged silica. One possible explanation for this result is that the freshly fractured silica yields ROS production through a pathway that is p53 independent. There is a significant rise in ROS production levels in cells with altered p53 expression as compared to wild-type cells following exposure to aged silica, suggesting the ROS production levels are inhibited in a p53-dependent manner following exposure to aged silica containing fewer reactive sites. Differences in ROS production of aged and freshly fractured silica and its correlation with AP-1 activation, an important transcription factor involved in neoplastic transformation, are well documented (Ding et al., 2001). These studies reported that freshly fractured silica was potent in activating AP-1, and after 2 wk of aging, potency declined only minimally, and subsequent to 8 wk, AP-1 activation potential declined to the 50% level. The results presented in this study also demonstrate that exposure to freshly fractured silica with more reactive sites may lead to enhanced ROS production through two pathways, one related to the surface active sites and one related to the consistent potency of silica. The variability in ROS production may also relate to altered apoptosis following exposure to silica in these three cell lines, as previous studies showed an increased apoptosis in cells with increased ROS production (Wang et al., 2007).

Increased levels of apoptosis were reported previously in cells exposed to oxidants derived from silica or asbestos (Hamilton et al., 1996; Janssen et al., 1998; Iyer et al., 1996). In a recent report it was also shown that both extrinsic (Fas-dependent), and intrinsic (mitochondria-dependent) pathways of apoptotic cell death are influenced by ROS (Wang et al., 2007). The apoptosis assay performed in this study monitored the early cleavage of internucleosomal DNA fragmentation that precedes many biochemical events such as capase activation, release of cytochrome *c*, externalization of phosphatidylserine, and polymerase polyribosome cleavage. These events occur prior to any detectable morphological changes. There was little to no apoptosis found in the wild-type p53 cell line either at baseline or following exposure to both freshly fractured or aged silica; however, both cancer cell lines showed an increased level in apoptosis. The p53 altered cell line showed the most significant rise in apoptosis following exposure to freshly fractured silica and with aged silica exhibited a significantly greater level of apoptosis (Figure 2). The normal cells and p53-deficient cells, on the other hand, displayed minimal differences in the level of apoptosis (Figure 2). These results suggest that there is an effect of p53 status on apoptosis following exposure to silica unrelated to ROS production. This effect may relate to temporal response to silica exposure, with the wild-type cells showing less apoptosis following 6 or 24 h of exposure to silica, while the cell lines with altered p53 expression show enhanced apoptosis. Comparing these results to those described earlier for ROS production, the apoptosis found in these cells does not appear to be correlated to ROS levels.

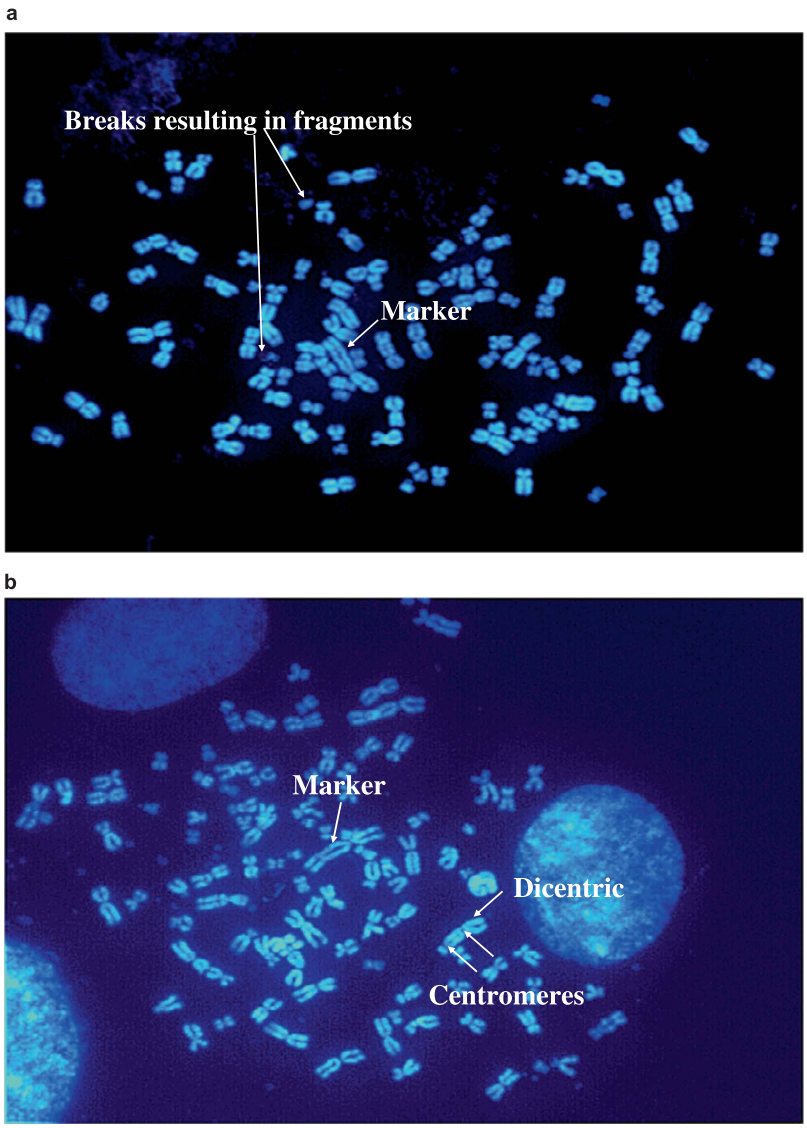

FIG. 5. Results of cytogenetic analysis demonstrated chromosome breakage and translocations in all cell lines; however, only the two tumor cell lines demonstrated statistically significant chromosome breakage. The normal cell line had a significant increase in dicentric chromosomes at 24 h. The number of dicentric chromosomes was much greater in the two tumor cell strains. (a) Representative image of chromosome fragments and a marker chromosome that were observed in 10% of the H460 cells after 24 h of exposure with freshly fractured silica. (B) The same marker chromosome in the H460 cell line after 24 h of exposure to freshly fractured silica; shown also is a chromosome with two centromeres (dicentric chromosome) that was present in 8% of the H460 cells. Values that were found to be statistically significant are indicated by an asterisk.

Subsequent to exposure to freshly fractured silica, p53 protein levels in three cell lines showed variable response. Generally, a stable p53 message is expected in cells with damaged or altered p53 protein. As measured by Western blot analysis, only the wild-type cell line showed a measurable level of p53 protein following 24 h of exposure to freshly fractured silica. There was no significant difference between treated and untreated cells, although the three cell lines clearly show varied levels of p53 regardless of exposure.

Molecular and cytogenetic characterizations of chromosomal changes and cell cycle are often linked to tumor development (Sargent et al., 2002). Cytogenetic and cell cycle analysis of all three cell lines showed some variability following exposure to freshly fractured or aged silica. The BEAS-IIB cell line, which expresses wild-type p53, displayed minimal alterations in cell cycle parameters with no alterations in cell ploidy. However, both of the cancer cell lines showed a rise in aneuploidy following exposure to silica, with the greatest increase found in cells exposed to freshly fractured silica as compared to aged silica (Figure 4). In addition, the p53 null cells had even more dramatic increase in aneuploidy when exposed to freshly fractured silica (98.8%) compared to aged

TABLE 2
Changes in Gene Expression (Fold Increases) Analysis Produced by Exposure to Freshly Fractured Silica for 24 h

	HBEAS	H460	H1299
p53 Binding Protein	3.21	0.48	329.99
ATM	3.31	0.81	10.98
SESN1	115.03	14.38	0.54
CDC25	15.84	0.78	2.68
MDM2	4.21	0.97	141.32
BCL6	9.72	2.12	708.67
BAX	4.10	0.57	247.41
PCNA	29.16	1.62	1.54
GADD45	0.65 (alpha)	−0.11 (alpha)	5.02 (alpha)
	0.00 (beta)	0.00 (beta)	4.65 (beta)
	6.98 (gamma)	0.65 (gamma)	1.47 (gamma)
ERCC3	10.36	0.56	2.56
RAD23A	2.46	11.43	1.41

Note. From a group of 104 genes analyzed for differences, in 7 genes expression of DNA damage signaling pathway genes showed variations potentially related to the different p53 expression in the three cell lines. These genes included p53 binding protein (p53BP1), ATM, MDM2, BCL6, PCNA, ERCC3, and RAD23. These gene expression differences as compared to untreated controls in the same cell line showed increases in these genes ranging from 2.12- to 708.97-fold. Select genes were further analyzed by RT-PCR to confirm the microarray results. RT-PCR results varied somewhat from those found on the array analysis. However, for the seven genes listed here, RT-PCR results confirmed alterations in gene expression as comparable to array analysis.

silica (61.9%). This rise in aneuploidy is suggestive of the potential of silica to transform cells and the important role of p53 in silica-induced carcinogenicity. The mechanisms involved in leading to carcinogenesis are unclear. However, it was recently shown that asbestos fibers induce aneuploidy and chromosome instability by binding to proteins involved in the regulation of cell cycle, cytoskeleton, and mitotic process, which is blocked by coating the fibers (MacCorkle et al., 2006). The results presented here show that the rise in aneuploidy in cancer cells occurs at an increased rate with the freshly fractured silica exposure. In these cancer cells with altered or dysfunctional p53 the second hit induced a significantly greater level of aneuploidy. This follows the two-hit hypothesis proposed by Knudson (1996) and would suggest that carcinogenicity related to silica exposure may occur more in susceptible populations with a previous genetic predisposition.

Further cytogentic analysis of chromosomal alterations, breakage, and translocation studies carried out corroborated with the findings of molecular changes observed in all cell lines. Minor alterations were seen in normal cells at 6 h following exposure, but by 24 h postexposure the majority of cells appeared normal. The most significant damage was seen in the H460 adenocarcinoma cell line, with increased chromosomal breakage as well as the introduction of a marker chromosome and endoreduplication by 24 h postexposure (Figure 5 and Table 1). This suggests an efficient DNA damage repair response in these cells was not functional as in other cancer cells. In parallel with the two-hit hypothesis, this indicates that in the case of silica-induced carcinogenicity, exposure to silica in cells with a deficient DNA repair pathway may lead to increased carcinogenicity.

Gene expression analysis focused on the DNA damage signaling pathways found variations in pathways that` were seemingly activated based on increased gene expression patterns. While the wild-type cells showed an increase in genes related to both base excision repair as well as nucleotide excision repair, the two cancer cell lines appear to activate only one of these pathways. Gene expression analysis also showed a rise in apoptotic genes (BAX) as well as antioxidant genes such as sestrin (Table 2). Figure 6 illustrates a schematic representation of potential pathways involved in the activation of genes following silica exposure. In this postulated pathway, ROS produced following exposure to freshly fractured silica may result in either direct DNA damage or activation of the AKT pathway. This activation of the AKT pathway is followed by the subsequent phosphorylation of MDM2, which in turn inhibits p53 by binding to the protein and ubiquination of p53. In the absence of MDM2, p53 would be phosphorylated by ATM, bind to p53-binding protein, and lead to induction of gene transcription of PCNA, Sestrin1, Bax and GADD45. DNA damage also may trigger the activation of the ATM pathway, which in turn results in the phosphorylation of proteins including GADD45 and p53. The p53 and MDM2 work by an autoregulatory loop by which positive and negative regulation of a feedback loop is controlled to govern important cellular events such as G1 arrest, apoptosis, and cell proliferation.

From the data presented from these cell studies, it may be conjectured that freshly fractured silica exposure may have an increased ability to produce cancer in susceptible populations, especially in those who succumbed to a previous genetic hit. The various cellular and molecular investigations used in this report showed consistent effects of freshly fractured silica that were in general increased in both cancer cell lines known to have previous damage related to a genetic alteration or deficiency. Data described here show that freshly fractured silica, as produced in certain occupations using mechanical tools, with increased levels of surface radicals may induce the second hit, resulting in enhanced aneuploidy, DNA damage, and upregulation of genes potentially leading to carcinogenesis, supporting a two-hit hypothesis.

The results of these cell studies confirmed that silica exposure may have an increased ability to produce cancer in susceptible populations, especially those populations having at least one previous genetic hit. However, the use of immortalized cells in this study precludes drawing direct conclusions on how freshly fractured silica exposure fits into the “second-hit”

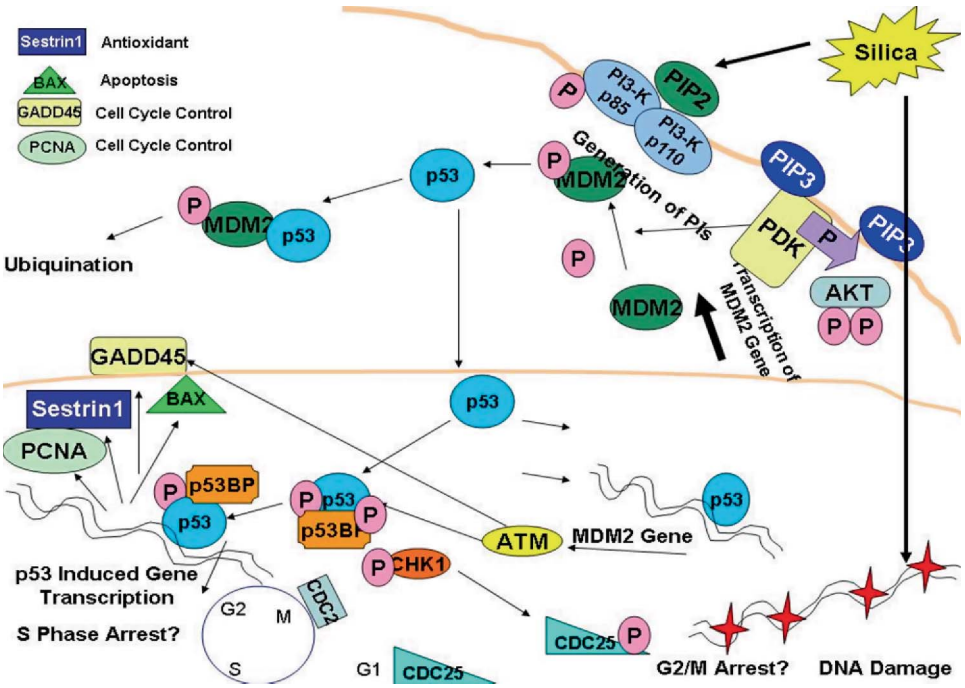

FIG. 6. Hypothetical schematic representation of activation of molecular events in response to freshly fractured silica exposure in human pulmonary cell lines based on gene expression patterns analyzed by Superarray DNA Damage Signaling Pathway array (OHS-029). Under this hypothesis, ROS produced following silica exposure may cause either direct DNA damage or activation of the AKT pathway, which can result in the phosphorylation of MDM2. Once phosphorylated, MDM2 inhibits p53 by binding to the protein and ubiquination of p53. In the absence of MDM2, p53 would be phosphorylated by ATM, bind to p53-binding protein, and lead to the induction of gene transcription of PCNA, Sestrin1, Bax, and GADD45. DNA damage also can trigger activation of the ATM pathway, which in turn leads to the phosphorylation of proteins including GADD45 and p53. Not shown in this figure is the upregulation of various DNA damage repair genes, as described in the text.

hypothesis. Data described here showed an elevation in enhanced ROS generation, increased DNA damage, aneuploidy, and chromosomal aberrations in cancer cells with altered or deficient p53 compared to the BEAS-IIB cells following exposure to silica. Since these lung cancer cells are already damaged, this suggests that the exposure to silica may have produced more damage to cellular machinery, or perhaps impaired DNA repair compared to the fully functional system in normal cells. An interesting aspect is the increased apoptosis observed in p53-altered cells compared to the other cancer cells with deficient p53 and normal cells. This may be related to greater DNA damage in both cancer cell lines and an efficient DNA repair mechanism still functional in p53-altered cells triggering apoptosis. Further support for this is also implied in the divergent gene expression results of MDM2 (141-fold), BCL6 (709-fold), BAX (247-fold) and the 330-fold increase in p53 binding protein in p53-deficient cells compared to a much lower level in other cancer cells and normal BEAS-IIB cells. Bcl-6 was found to decrease ROS production and apoptosis, which may explain some of the different responses observed between the two cancer cell lines (Kurosu et al., 2003). Overall evidence indicates that freshly fractured silica produced in certain occupations using mechanical tools contains elevated levels of surface radicals, which may promote increased adverse health effects, inducing DNA damage, increased aneuploidy, apoptosis, and upregulation of genes potentially leading to carcinogenesis. A follow-up study using normal cells and comparing with a knock-in mutant p53 gene (one-hit) may be valuable to unravel the intricate mechanistic events underlying the second-hit hypothesis.

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