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Micronucleus formation in V79 cells treated with respirable silica dispersed in medium and in simulated pulmonary surfactant

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Abstract

Chinese hamster lung fibroblasts (V79 cells) were challenged with respirable silica particles using an in vitro genotoxicity assay. Two particle sizes of crystalline quartz and a non-crystalline silica were assayed for induction of micronuclei (MN) in V79 cells. Some of the silica dusts used were pretreated with simulated pulmonary surfactant to model in vivo exposure conditions. The results showed that both crystalline and non-crystalline silica dispersed in medium (MEM) induced MN formation in a dose-dependent manner. Crystalline silica was more active in this assay than non-crystalline silica on a mass basis. The results also show that the frequency of micronucleated cells in cultures treated with surfactant-coated silica was not significantly different from that of the non-treated control cultures. These results seem to indicate that silica can cause chromosomal aberrations and/or aneuploidies in V79 cells; however, pretreatment of silica particles with simulated pulmonary surfactant reduces or delays genotoxicity in this assay.

Keywords: Silicon dioxide; Quartz; V79 lung fibroblast; Micronucleus induction; Pulmonary surfactant; Dipalmitoylphosphatidylcholine

1. Introduction

While crystalline silica is a well-known cause of fibrotic lung disease in humans, the link between exposure to crystalline silica and lung cancer is less clear. Both animal exposure studies and epidemiology studies have addressed this question. Inhalation studies have demonstrated that crystalline silica is carcinogenic in rats [1]. In humans, the association between occupational exposure to crystalline silica and respiratory cancer is probable but still controversial [2]. A few limited in vitro studies have been reported; however, inconsistent results have led to no definite conclusions about the genotoxic potency of crystalline silica [3].

When respirable crystalline silica is inhaled to the alveolar region and deposited in the lung, it first contacts the pulmonary surfactant layer that forms the alveolar/air interface and becomes coated with surfactant components. Since this interaction with surfactant precedes any tissue interaction, this step may be important in the initial stages of disease

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induction. Thus surfactant/silica particle association should be a feature of in vitro studies.

Pulmonary surfactant is a phospholipid-rich mixture of proteins and lipids with dipalmitoylphosphatidylcholine (DPPC) as a major component [4]. Pulmonary surfactant constitutes the hypophase that lines the inner surface of the bronchoalveoli and is a surface active material reducing surface tension in the lung [4]. A few in vitro experiments [5–8] have been performed to determine the cytotoxic differences between surfactant-coated quartz and native quartz, with the findings indicating that surfactant-coated quartz is less cytotoxic than native quartz. Because little attention has been paid to the effects of surfactant on genotoxicity, two important questions are raised: (1) could surfactant treatment increase the genotoxicity of quartz particles in short-term exposures by decreasing cytotoxicity and by making uptake by cells easier, and (2) if surfactant does modify uptake and cytotoxicity of quartz particles, are surfactant components removed after uptake, and will genotoxicity be expressed after a time delay.

In this experiment, DPPC was used to model pulmonary surfactant in vitro. The MN formation test, which is sensitive to genotoxic chemicals and widely used to determine genotoxicity, was employed in Chinese hamster lung fibroblasts (V79 cells) to study the genotoxicity of native and surfactant-coated quartz. In the first part of our studies, V79 cells were challenged with two crystalline silicas and a non-crystalline silica. In the second part, two DPPC-coated crystalline silicas were incubated with V79 cells for various exposure times to study the effect of surfactant on the quartz-induced MN formation.

2. Materials and methods

DPPC (5 mg/ml) dispersion was prepared by adding 200 mg of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (CAS 63-89-8) (Calbiochem, La Jolla, CA) to 40 ml of Eagle's MEM (Sigma) without serum in a 50 ml centrifuge tube. This was followed by sonication for 10 min at 15% power (72 W) with an ultrasonic processor equipped with a microprobe tip (Heat Systems, Farmingdale, NY).

Crystalline silica suspensions were Min-U-Sil 5 (96 mg/ml) and Min-U-Sil 10 (96 mg/ml) (U.S. Silica, Berkeley Springs, WV), prepared by weighing 800. mg of dusts into glass bottles, steam autoclaving for 20 min at 121°C and adding 8.3 ml of complete medium (MEM + 1% penicillin/streptomycin (Sigma, St. Louis, MO) + L-glutamine (Sigma) + 10% fetal bovine serum (heat denatured) (Sigma) and vortexing.

Amorphous silica (SSB1) (93 mg/ml) was prepared by adding 1000 mg of 5 μm Spherisorb® amorphous silica (Phase Sep, Norwalk CT) to a glass bottle and autoclaving 20 min at 121°C, adding 10.8 ml of MEM and vortexing.

Suspensions of Min-U-Sil 5 and Min-U-Sil 10 dispersed in DPPC were made by adding 1000 mg of the respective dusts to glass bottles and autoclaving as above, adding 20 ml of 5 mg DPPC/ml in MEM, transferring to centrifuge tubes, and incubating for 2 h at 37°C in a rotary drum incubator. Samples were removed, spun 10 min at $1000 \times g$, the supernatant discarded, 10.4 ml complete medium added, and the suspension was vortexed.

All stock silica suspensions were used undiluted or diluted 1 : 2, 1 : 4 and 1 : 8 with complete medium; and 0.1 ml of the desired diluted silica suspension was added to 9.9 ml of medium in the culture dish. Culture dishes had a useable area of approx. 58 cm^2. Final challenge concentrations were 20, 40, 80 and 160 $\mu g/cm^2$.

3. Treatments

Short-term challenge. 5.0×10^5 V79 cells were seeded into each 100 mm dish with 10 ml of complete medium. The dishes were incubated at 37°C and 5% CO_2 for 24 h. The cells were then challenged with native Min-U-Sils 5 and 10, native SSB1, and DPPC-coated Min-U-Sils 5 and 10 at the desired concentrations for 24 h.

Extended time challenge. 1.0×10^5 V79 cells were seeded into each 100 mm dish with 10 ml of complete medium. After 24 h of incubation at 37°C and 5% CO_2, DPPC-coated Min-U-Sils 5 and 10 were added at a final concentration of 80 $\mu g/cm^2$. The treatment lengths were 1, 3 and 5 days, with two sets carried out simultaneously. In set 1, at the

second and fourth days of exposure, medium was transferred into sterile tubes and centrifuged at 1000 $\times g$ for 10 min. The supernatant was removed and then the particles resuspended in 10 ml of fresh medium and transferred back into the original dish for the remainder of the incubation. In set 2 the medium remained unchanged throughout the treatment.

3.1. Micronucleus assays

After treatment, the medium was removed and the cells were rinsed 8 times with 5 ml of phosphate-buffered saline (PBS) (pH 7.2), followed by 24 h of post-treatment incubation. Cells were harvested by trypsinization and slides were prepared using a Cytospin® (Shandon, Pittsburgh PA). Slides were fixed in methanol for 10 min and then stained with Diff-Quik® solution (American Scientific Products, McGaw Park, IL). The slides were coded and 3000 cells from each treatment were scored for micronuclei. All of the experiments were repeated at least two times. The Chi-squared test and linear regression were used for statistical analysis.

4. Results

Micronuclei induced by native silica treatment for 24 h. Table 1 shows the MN formation induced by Min-U-Sils 5 and 10 and SSB1 in V79 cells. The two native crystalline silica samples and the one native amorphous silica sample induced MN formation in V79 cells, while high cytotoxicity was observed at the inducing concentrations. The MN frequencies were significantly elevated over their respective solvent controls in a dose-dependent manner. The correlation coefficients for Min-U-Sils 5 and 10 and SSB1 were 0.95, 0.95 and 0.96, respectively.

Micronuclei formation in V79 cells treated with DPPC-coated silica for 24 h. Table 2 presents the results of MN formation in V79 cells treated with DPPC-coated Min-U-Sils 5 and 10. Data show that the DPPC-coated particles failed to induce MN formation at all concentrations when compared to the uncoated particles. Less cytotoxicity was evident for the DPPC-treated particles relative to the untreated silica particles.

Table 1
Frequency of micronuclei induced by silica particles in V 79 cells

Compound	Concentration ($\mu g/cm^2$)	Frequency of MN ($\bar{x} \pm SE$)
Min-U-Sil 5	0	6.67 ± 0.33
	20	9.33 ± 0.88
	40	15.67 ± 0.88 [b]
	80	20.67 ± 1.77 [b]
	160	25.67 ± 0.33 [b]
Min-U-Sil 10	0	6.67 ± 0.33
	20	9.67 ± 0.67
	40	11.67 ± 0.67 [a]
	80	17.00 ± 1.00 [b]
	160	19.67 ± 0.67 [b]
SSB1	0	7.67 ± 2.33
	20	10.33 ± 0.86
	40	8.67 ± 2.41
	80	13.33 ± 1.77 [a]
	160	18.00 ± 2.08 [b]
MNNG [c]	1 (μg/ml)	96.67 ± 9.53 [b]

3000 cells were scored for each treatment group; frequencies are mean values per 1000 cells.
[a] Compared with solvent control $p < 0.05$.
[b] Compared with solvent control $p < 0.01$.
[c] MNNG was used as a positive control in this experiment.

Table 2
Frequency of micronuclei induced by DPPC-coated silica in V 79 cells

Compound	Concentration ($\mu g/cm^2$)	Frequency of MN ($\bar{x} \pm SE$)
DPPC		9.00 ± 1.16
Min-U-Sil 5 + DPPC	20	10.67 ± 0.88
	40	13.33 ± 1.70
	80	10.67 ± 1.86
	160	11.00 ± 0.58
Min-U-Sil 10 + DPPC	20	15.67 ± 0.67
	40	10.67 ± 2.34
	80	10.67 ± 2.34
	160	11.33 ± 1.16
MNNG [a]	1 μg/ml	141.00 ± 23.71

3000 cells were scored for each treatment group; frequencies are mean values per 1000 cells.
[a] MNNG was used as a positive control in this experiment.

Table 3
Frequency of micronuclei induced by DPPC-coated quartz particles for varying exposure times

Chemical	Exposure length (days)	Frequency of micronuclei	
		Set I[a]	Set II [b]
MEM	1	6.33 ± 1.33	7.67 ± 1.20
	3	5.00 ± 0.00	8.00 ± 0.58
	5	5.33 ± 0.67	22.67 ± 2.34 [c]
DPPC	1	6.33 ± 0.88	7.33 ± 0.88
	3	6.00 ± 1.00	9.33 ± 0.88
	5	6.00 ± 0.00	21.33 ± 3.18 [c]
Min-U-Sil 5 + DPPC ($80\ \mu g/cm^2$)	1	5.67 ± 1.20	7.67 ± 2.19
	3	6.00 ± 2.00	8.33 ± 1.33
	5	6.67 ± 0.88	21.33 ± 0.67 [c]
Min-U-Sil 10 + DPPC ($80\ \mu g/cm^2$)	1	5.00 ± 0.58	6.33 ± 0.67
	3	3.67 ± 0.67	8.33 ± 0.33
	5	6.33 ± 0.67	23.67 ± 2.41 [c]

3000 cells were scored for each treatment group; frequencies are mean values per 1000 cells.
[a] In Set I the medium was changed on the 2nd and 4th days of exposure.
[b] In Set II the medium was not changed during the exposure.
[c] $p < 0.01$ compared with respective day 1 control.

Genotoxicity of DPPC-coated silica particles with prolonged treatment time. In order to observe the possibility of genotoxic properties of DPPC-coated silica particles with prolonged treatment, DPPC-coated Min-U-Sils 5 and 10 were studied at the concentration of 80 $\mu g/cm^2$ for various exposure times. The results (Table 3) showed no increased MN formation in V79 cells treated with silica particles for 1, 3 or 5 days with medium changes on the second and fourth days. However, in set 2, all of the 5-day treatment groups (blank control, DPPC solvent control, Min-U-Sils 5 and 10) incurred a significant increase in MN frequencies over their respective 1-day treatment groups (Table 3).

5. Discussion

A number of in vitro experiments studying cytotoxicity of quartz with and without surfactant treatment have been reported [5,9,7,8,10] with consistent positive results. Recent theories suggest that quartz damage of biological membranes contributes to that mineral's cytotoxicity [8]. Research to date, however, has not detailed unequivocally the in vitro genotoxicity of quartz. Oshimura et al. [11] reported that neither chromosomal aberration nor cellular transformation occurred in Syrian hamster embryo (SHE) cells treated with quartz at the concentration of 2.0 $\mu g/cm^2$. Price-Jones et al. [12] failed to observe the sister-chromatid exchange and chromosomal abnormalities in V79 cells challenged with Min-U-Sil quartz at a dosage of 15 $\mu g/ml$. A study by Hesterberg and Barrett [13] showed that Min-U-Sil quartz and α-quartz induced cell transformation in SHE cells at doses greater than 2 and 10 $\mu g/cm^2$, respectively, although the relative survival of the cells decreased. Pairon et al. [3] ran four SCE assays with lymphocytes and monocytes using different concentrations of quartz; even at the highest concentration of 50 $\mu g/cm^2$ a significant increase in SCE was observed only once. In this study, V79 cells were treated with two crystalline silica quartz dusts and one non-crystalline silica. All non-treated crystalline and non-crystalline silicas tested enhanced the induction of micronuclei. Cytotoxicity was noted at all concentrations. Min-U-Sils 5 and 10 are different sized quartz dusts; the smaller Min-U-Sil appears to be a stronger inducer of MN. Crystalline silica was more genotoxic than non-crystalline silica on a mass basis.

Compared with other studies reported in the literature, the dust concentrations used in this study were much higher. At doses lower than 40 $\mu g/cm^2$, all three silica dusts failed to significantly increase the frequency of MN formation. This suggests that native silica is a weak inducer of MN in V79 cells. Further work is needed to determine whether MN induced by silica is due to clastogenicity and/or aneuploidy.

It has been reported that the ability of quartz coated with lavaged or simulated surfactant to injure cells in vitro is much less than that of uncoated quartz [5–10]. This protective effect of surfactant is due to direct interaction between the surfactant and the surfaces of the quartz [8]; which can absorb phospholipids on its surface. In this assay, only Min-U-Sil 5 and Min-U-Sil 10 were coated with

DPPC to study the effects of surfactant on quartz genotoxicity. The results showed that at all concentrations prepared, DPPC can prevent MN formation. This protective effect may be due to DPPC modification of the quartz surface.

In our previous work [6,14], a cell-free enzyme phospholipase A_2 in vitro system was used to remove dipalmitoyl lecithin absorbed on quartz; the cytotoxicity of DPL-coated quartz was restored after digestion in that cell-free system. Hill et al. [15] measured the in vitro cellular digestion of silica-adsorbed DPPC in $P388D_1$ cells. According to Antonini and Reasor [10], rat alveolar macrophages (RAMs) treated with Survanta®-coated silica and incubated for 24 h exhibited significantly increased cytotoxicity relative to those incubated for one hour. The question arises as to whether the metabolism of DPPC can affect the genotoxicity of DPPC-coated quartz. In the present study, we treated V79 cells with DPPC-coated Min-U-Sils 5 and 10 at a concentration of 80 $\mu g/cm^2$ for 1, 3 and 5 days, respectively. The results showed that DPPC coated Min-U-Sils 5 and 10 did not induce MN formation after exposure of cells for 1, 3 or 5 days, in which medium was changed. In a second set of samples, DPPC coated Min-U-Sils 5 and 10 incubated with cells for 5 days in unchanged MEM showed significantly increased frequencies of MN formation over their 1 day groups. There was, however, no significant difference between treatment groups and control groups (blank or solvent control). Therefore, the increase of MN formation in 5 day exposure groups with unchanged MEM may be due to changes in culture medium composition rather than the genotoxicity of coated particles.

In this study, mammalian cells in short-term in vitro assays were challenged with quartz and non-crystalline silica particles and this resulted in a weak but significant dose-dependent induction of micronuclei. Micronucleus induction was not induced when the dusts were pretreated with simulated pulmonary surfactant and maintained up to 5 days. Other research has shown that surfactant adsorption by silica dusts suppresses prompt direct membranolytic activity. These results suggest questions about the mechanisms of micronucleus induction by silica. Clastogenic or aneuploidogenic damage might be due to direct interactions of particle surfaces with genetic material or the spindle apparatus. Or, micronucleus induction might be a secondary or indirect result of other cytotoxic processes. Surfactant suppression of prompt particle-induced membranolysis would suppress expression of an indirect genotoxic activity which is a consequence of that prompt cytotoxicity. However, the suppression of short-term micronucleus induction seen in this study does not necessarily imply an indirect mechanism. Examination of micronucleus induction over a longer time and comparison with the time course of restoration of surfactant-treated silica cytotoxicity may help to elucidate the possible genotoxic mechanisms of silica under conditions of deposition in the lung.

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