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Effects of Phospholipid Surfactant on Apoptosis Induction by Respirable Quartz and Kaolin in NR8383 Rat Pulmonary Macrophages

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Apoptosis was measured in rat alveolar macrophage NR8383 cells challenged *in vitro* with respirable quartz or kaolin dust and with the dusts pretreated with dipalmitoyl phosphatidylcholine (DPPC) to model conditioning of respired dusts by interaction with a primary phospholipid component of pulmonary surfactant. Quartz dust is known to induce apoptosis *in vitro* and *in vivo*. For this study, quartz and kaolin were compared as dusts of similar cytotoxicity in some *in vitro* assays but of differing pathogenic potential: quartz can cause significant pulmonary fibrosis while kaolin generally does not. NR8383 cells exposed to native quartz at concentrations from 50 to 400 μg/ml for 6 h showed a dose-dependent increase in apoptosis measured by the TdT-mediated dUTP-fluorescein nick end labeling (TUNEL), cell death ELISA, and DNA ladder formation assays, while native kaolin induced significant response only at the higher concentrations and only in the TUNEL and ELISA assays. For cell challenge from 6 h to 5 days at 100 μg/ml of dust, quartz was active at all times while kaolin was active only at 5 days. DPPC pre-treatment suppressed quartz activity until 3 days and kaolin activity through 5 days. Cellular release of lactate dehydrogenase, measured in parallel experiments to compare dust apoptotic and necrotic activities, indicated that components of serum as well as surfactant may affect kaolin *in vitro* expression of those activities.

Key Words: **apoptosis; cell death ELISA assay; dipalmitoylphosphatidylcholine; DNA ladder formation assay; kaolin; lactate dehydrogenase assay; pulmonary fibrosis; pulmonary surfactant; quartz; rat macrophage NR8383; respirable dust; serum; surfactant; TUNEL assay.**

Exposure to silica dust can result in lung inflammation, fibrosis, and cancer (IARC, 1997). Exposures to respirable kaolin aluminosilicate dust are not known to present such significant risk for pulmonary disease. In workers exposed to kaolin dust during the milling and bagging of kaolin, there was an increased prevalence of pneumoconiosis, however, kaolin clay mineral-induced disease typically was not as severe as that induced by quartz (Schulz, 1993). Seemingly counter to this, *in vitro* membranolysis or cytotoxicity assays have found kaolin dust and some other nonpathogenic dusts to express cytotoxic activity comparable to that of quartz dust, as measured by several cellular assays, e.g., LDH, β-glucuronidase, and β-*N*-acetyl glucosaminidase release. (Vallyathan *et al.*, 1988). Therefore quartz and kaolin dusts are being compared for their *in vitro* activities that might identify bases for their apparent differences for disease risk. Quartz is known to be active for apoptosis induction (Leigh *et al.*, 1997); the current study provides a direct comparison of kaolin. Dusts are compared both in their native state and after incubation in the primary phospholipid component of pulmonary surfactant. This is to model, in part, the conditioning of the surfaces of particles depositing in the deep lung bronchioles or alveoli.

Mechanisms involved in the development of silica-induced pathological changes have not been fully defined. When respirable particles or fibers deposit in a pulmonary alveolus, they interact with the pulmonary fluids coating the epithelial cell surface. Previous studies have shown that dipalmitoyl phosphatidylcholine (DPPC) is adsorbed from dispersion in physiological saline by quartz and kaolin particles and suppresses the otherwise prompt *in vitro* cytotoxicity of the dusts (Wallace *et al.*, 1985). Acellular studies have shown that phospholipase enzymes can digest DPPC from the dusts, with a consequent restoration of cytotoxic activity (Wallace *et al.*, 1988, 1992). *In vitro* studies have found that cellular processes can digest quartz- and kaolin-adsorbed DPPC (Hill *et al.*, 1995) and can restore quartz toxicity (Liu *et al.*, 1998). Some observations indicate that pulmonary alveolar or interstitial macrophages play a central role in the development of inflammation or fibrosis (Flint, 1988; Martin *et al.*, 1984; Adamson *et al.*, 1989). When exposed to silica particles, various cytokines, growth factors, and free radicals are generated in the lung (Rom, 1991; Blackford *et al.*, 1994; Piguet *et al.*, 1990; Williams *et al.*, 1993) and are thought to play an important role in the development of silica-induced inflammation. In addition to silica per se, some of these substances may induce apoptosis. Apoptotic cells are frequently observed in bronchoalveolar

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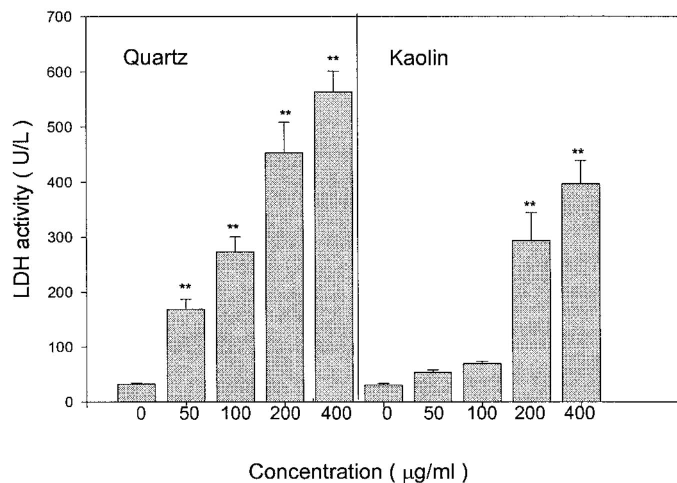

FIG. 1. Lactate dehydrogenase activity induced by quartz and kaolin in NR8383 rat alveolar macrophages (AM) after challenge for 6 h. Significant LDH activities were observed in both quartz and kaolin-treated AM. Values represent the mean LDH activity (U/L) ± SE for five replications of this experiment. **Statistical significance comparing quartz or kaolin to control as determined by Dunnett's t test, $p < 0.01$.

lavage fluid in silica-induced pneumoconiosis (Leigh *et al.*, 1997). Apoptosis is a mechanism of cellular death believed to play an important role in a wide variety of physiological conditions (Thompson, 1995) and in the resolution of inflammatory reactions. Shrinkage of cells accompanied by unique DNA fragmentation, about 180–200 bp, caused by the activation of an endonuclease, are characteristic features of a cell undergoing apoptosis. Deregulation of apoptosis may contribute to the pathogenesis of many diseases, ranging from cancer to acquired immunodeficiency syndrome (Waring *et al.*, 1991).

The purposes of this study were to determine whether respirable-sized kaolin as well as quartz particles could induce apoptosis in NR8383 rat pulmonary macrophages *in vitro*; to measure the effect of DPPC surfactant pretreatment of the dusts on the apoptotic response; and to compare this with dust-induced necrosis. Apoptosis was evaluated by cell morphology, TdT-mediated fluorescein nick end labeling (TUNEL), cell death ELISA, and DNA ladder formation assays; necrosis was assayed by cellular release of lactate dehydrogenase (LDH).

MATERIALS AND METHODS

Mineral dusts. Min-U-Sil 5 respirable quartz dust (U.S. Silica Corporation, Berkeley Springs, WV) was determined by X-ray diffraction to be 99.5% alpha quartz with 98% of particles smaller than 5 μm area equivalent diameter. The specific surface area of this quartz dust was 3.97 m^2/g as measured by BET N_2 gas adsorption (Brunauer *et al.*, 1938). A sized fraction of respirable kaolin dust (Georgia Kaolin Mills, Augusta, GA) used was at least 95% aluminosilicate with no crystalline quartz detected by X-ray diffraction, with 99% of the fraction < 5 μm area equivalent diameter. The specific surface area of this kaolin dust was 13.25 m^2/g as measured by BET N_2 gas adsorption.

Surfactant. Dipalmitoyl phosphatidylcholine (Calbiochem, San Diego, CA) was ultrasonically dispersed into 0.165 M NaCl physiologic salt solution (PSS), at 5 mg DPPC/ml PSS, followed by centrifugation at 1500g for 10 min to remove nondispersed DPPC. Quartz and kaolin were mixed in this disper-

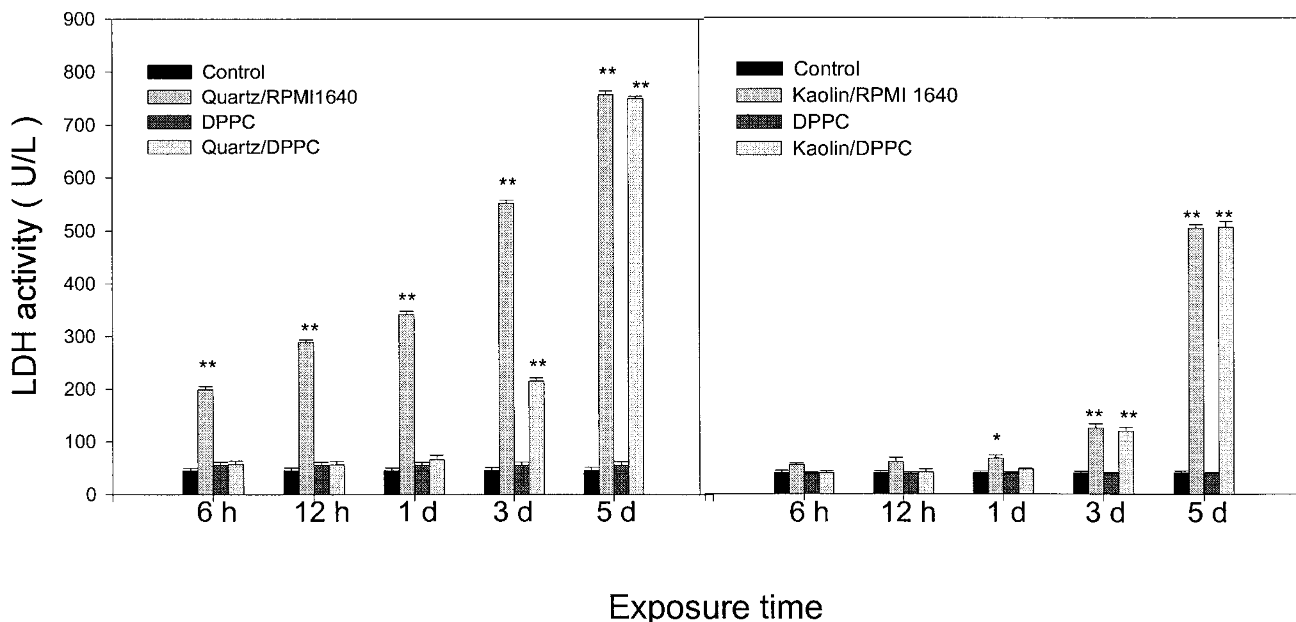

FIG. 2. Time course of untreated and DPPC-treated quartz and kaolin expression of LDH activity in NR8383 rat alveolar macrophages after exposure to 100 μg/ml of dust. Controls were cells in growth medium only. Values represent the mean LDH activity (U/L) ± SE for five replications of this experiment. Statistical significance comparing untreated or DPPC-treated quartz to control as determined by Dunnett's t test at $*p < 0.05$, $**p < 0.01$.

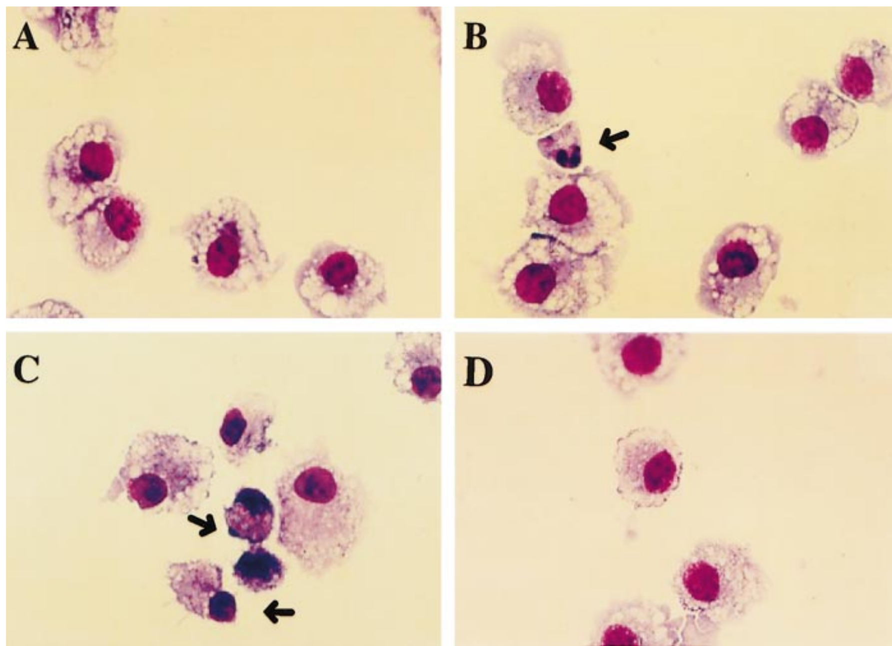

FIG. 3. Photomicrographs of Diff-Quik-stained NR8383 rat alveolar macrophages after treatment with quartz or kaolin: (A) control, (B) 100 μg/ml quartz, (C) 200 μg/ml quartz, and (D) 100 μg/ml kaolin. Cells were challenged with quartz or kaolin for 6 h. Quartz caused apoptosis in rat AM at 50 to 400 μg/ml, whereas kaolin did not induce apoptosis at 50 to 200 μg/ml and caused limited apoptosis at 400 μg/ml. Apoptotic cells (arrows) have a dark, condensed, segmented nucleus. Magnification 1000× (oil) in bright field.

sion at a ratio of 0.1 g DPPC/g quartz and 0.2 g DPPC/g kaolin and then centrifuged at 1500*g* for 10 min. The supernatant was discarded, and the dusts were resuspended in complete RPMI 1640 medium to desired concentrations. Prior studies of these preparations had shown that the quartz adsorbs about 60 mg DPPC/g and the kaolin adsorbs about 150 mg DPPC/g as multilayers. Approximately 20 mg DPPC/g quartz and 80 mg DPPC/g kaolin provides a bilayer covering that is stable to rinsing and fully suppresses hemolytic activity (Wallace *et al.*, 1992).

Cell culture. NR8383 (American Type Culture Collection, ATCC, Manassas, VA) is a rat alveolar macrophage cell line derived by lung lavage of a normal adult male Sprague–Dawley rat. NR8383 cells were maintained as a monolayer culture in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10% heat-denatured fetal bovine serum, and 1% penicillin–streptomycin solution. The cells were cultured at 37°C in a humidified incubator with 5% CO_2.

Five million cells were seeded in each well of a 6-well plate for cell viability, morphology, TUNEL, and DNA ladder assays, and 1×10^5 cells in 0.1 ml medium were seeded into each well of a 96-well plate for ELISA assay. All cultures were incubated at 37°C and 5% CO_2 for 24 h to permit cell adherence; and culture medium then was removed by suction. Cells were challenged for 6 h with untreated and DPPC-treated quartz and kaolin concentrations of 50, 100, 200, or 400 μg/ml; and cells were incubated at a dust concentration of 100 μg/ml for selected times of 6 h to 5 days.

Cytotoxicity analysis. Cell damage was determined by measuring LDH activity in the culture media using an LDH assay kit (Roche Diagnostics, Indianapolis, IN).

Cell morphology. Seventy-five microliters of cell suspension was placed on a cytospin slide and centrifuged at 600 rpm for 7 min (Shandon, Pittsburgh, PA), fixed in cold methyl alcohol for 5 min, and stained with Diff-Quik (Dade AG, Miami, FL). The slides were air dried and examined by light microscopy under oil immersion (1000×). At least two slides were made for each sample.

TUNEL assay. Apoptosis was determined by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-fluorescein nick end labeling assay, which measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3′-OH DNA ends using the enzyme TdT. The fluorescein-12-dUTP-labeled DNA can then be visualized directly by fluorescence microscopy. A 75-μl cell suspension aliquot (1×10^6 cells/ml) was

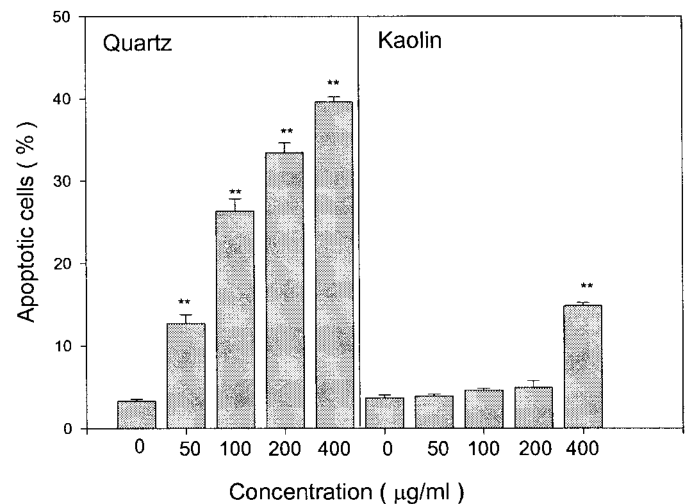

FIG. 4. Apoptotic cells induced by quartz and kaolin in NR8383 rat pulmonary macrophages after challenge for 6 h using fluorescein assay. Values represent the mean apoptotic cells (%) ± SE for five replications of this experiment. **Statistical significance comparing quartz or kaolin to control as determined by Dunnett's *t* test at $p < 0.01$.

placed on a cytospin slide and centrifuged at 600 rpm for 7 min and fixed by immersing slides in freshly prepared 4% methanol-free formaldehyde solution in PBS for 25 min at 4°C, with repeat washing of the slides by immersion in fresh PBS for 5 min at room temperature. Cells were permeabilized by immersing the slides in 0.2% Triton X-100 solution in PBS for 5 min. Slides were rinsed with PBS for 5 min, covered with 100 μl of equilibration buffer for 5–10 min at room temperature, and, after removal of the buffer, incubated with 50 μl of TdT incubation buffer for 60 min at 37°C inside a humidified chamber. The reaction was terminated by immersion of the slides in sodium chloride and sodium sulfate for 15 min at room temperature. The slides were washed three times with PBS for 5 min at room temperature. Cells were stained by immersing the slides in 40 ml of 1 μg/ml propidium iodide in PBS for 15 min at room temperature in the dark. Slides were washed three times with deionized water for 5 min, drained, and a drop of Anti-Fade solution was added to the area containing the treated cells. Cells were mounted using glass coverslips and were analyzed immediately under a fluorescence microscope using a standard fluorescein filter set to view the green fluorescence of fluorescein at 520 ± 20 nm and the red fluorescence of propidium iodide at >620 nm.

Cell death ELISA assay. For the detection of oligonucleosomes in cytoplasmic fractions of the cells, the samples were processed and analyzed with the Cell Death Detection enzyme-linked immunosorbent assay kit (Boehringer Mannheim, Indianapolis, IN) using monoclonal antibodies directed against DNA and histones. Cells (1×10^5) from each sample were processed; 5000 cells were used for each reaction, and triplicate reactions were performed for each sample. Cell medium was removed and washed with incubation buffer three times, and rinses were removed. Lysis buffer (200 μl) was added to each well and incubated for 30 min at room temperature. The lysate was centrifuged at 200*g* for 10 min and 20 μl of supernatant was transferred into the streptavidin-coated microtiter plate (MTP) for analysis. The immunoreagent mix, containing anti-DNA-peroxidase and anti-histone-biotin, was added to each well (80 μl), the MTP was covered with an adhesive cover foil, and the plate was incubated for 2 h at room temperature. The solution was removed thoroughly by tapping, rinsing wells three times with 250–300 μl of incubation buffer per well, and the washing buffer solution was removed carefully. 2,2′-Azino-di[3-ethylbenzthiazolin-sulfonate] (ABTS) substrate solution (100 μl) was pipetted into each well and incubated for 5 min. Absorbance was measured at 405 nm against substrate solution as a blank.

DNA ladder assay. The samples were analyzed using the Suicide-Track DNA Ladder Isolation Kit (Oncogene Research Products, Cambridge, MA) according to the manufacturer's protocol. Cell suspensions (5×10^5 to 1×10^6 cells) were transferred to a microcentrifuge tube and centrifuged at 1000*g* for 5 min at room temperature. The supernatant was removed, and the cell pellet was resuspended in 500 μl extraction buffer, incubated for 30 min on ice, and centrifuged at 15,000–16,000*g* for 5 min at room temperature. The supernatant was carefully removed and transferred to a clean tube. A solution (20 μl) for the degradation of RNA in the cell lysate was added; samples were incubated at 37°C for 60 min; 25 μl of solution for isolation of DNA from the cell lysate was added and mixed gently; and the samples were incubated at 50°C overnight. Pellet Paint Coprecipitant (2 μl), 60 μl 3 M sodium acetate, pH 5.2, and 662 μl 2-propanol were added; the solution was mixed by inversion and incubated at room temperature for 2 min and then centrifuged at 15,000–16,000*g* for 5 min. The supernatant was removed and the pellet was rinsed with 500 μl 100% ethanol and centrifuged at 15,000–16,000*g* for 5 min. The supernatant was removed and the pellet was air dried by placing the inverted tube open on the benchtop for a few minutes at room temperature and resuspended in 50 μl of resuspension buffer. The DNA ladder sample (20 μl) was transferred to a clean centrifuge tube; 2 μl loading buffer was added and the sample was loaded onto the gel. Gel electrophoresis was run at 5 V/cm for 2 h in TAE buffer (Tris base, acetic acid, and EDTA) containing 0.5 μg/ml ethidium bromide. DNA ladders were visualized by UV illumination.

Statistical analysis. Values are presented as means ± SE. Statistical differences between control and treated groups were determined by Dunnett's *t* test to take into account that the *t* tests are correlated, which increases the statistical power, and also to control for the multiple comparisons, which lowers the power. Differences were considered statistically significant at $p \leq 0.05$; significance at $p \leq 0.01$ also was calculated.

RESULTS

Quartz and Kaolin-Induced Macrophage Injury

Quartz and kaolin caused a dose-dependent increase in LDH release from NR8383 cells (Fig. 1). Exposure to 50, 100, 200, or 400 μg/ml of untreated quartz for 6 h resulted in statistically significant LDH release above the control, with a monotonic increase in LDH release with increasing quartz concentration. Cell challenge for 6 h with native kaolin resulted in significant LDH release at the two highest kaolin concentrations of 200 and 400 μg/ml; response was not significant at the lower kaolin concentrations. At the two highest dust concentrations, native kaolin expressed about two-thirds the activity of native quartz in this LDH assay.

Figure 2 shows that challenge of cells over a 5-day period with native quartz or with native kaolin at 100 μg/ml caused a time-dependent increase in LDH release, which was significant at $p < 0.01$ for quartz at all time points and was significant for kaolin at $p < 0.01$ at 3 and 5 days. DPPC treatment delayed significant quartz-induced LDH activity until 3 and 5 days. By 5 days the effect of DPPC treatment was no longer seen.

Quartz and Kaolin-Induced Apoptosis in NR8383 Cells

Morphological indications of apoptosis in NR8383 cells were seen in cells 6 h after challenge by quartz at 50 to 400 μg/ml and by kaolin at 400 μg/ml. Figures 3A–D are representative photomicrographs (1000×) of cells incubated for 6 h with medium control (A), 100 μg/ml quartz (B), 200 μg/ml quartz (C), and 100 μg/ml kaolin (D), respectively. Control cells are rounded with uniformly large, light purple nuclei and normal cytoplasm. In contrast, Figs. 3B and 3C show quartz (100 and 200 μg/ml)-treated cells with dark, shrinking nuclei indicative of nuclear condensation common to apoptosis. Nuclear disintegration is also apparent in some cells, characterized by a dark, condensed, segmented nucleus. Figure 3D shows that cells exposed for 6 h to kaolin at 100 μg/ml concentration have a morphology consistent with control cells, with no apparent apoptotic cells.

Figure 4 shows the proportion of apoptotic cells (%) induced by quartz and kaolin in NR8383 cells after exposure for 6 h at dust concentrations from 50 to 400 μg/ml as measured by the TUNEL fluorescein assay. (Leigh *et al.*, 1997). Native quartz challenge caused a statistically significant increased percentage of apoptotic cells above background at all native quartz dust concentrations and percentage of apoptotic cells increased with

increasing dust concentration. Kaolin induced a significant response only at the highest concentration (400 μg/ml). Representative illustrations of these TUNEL assay data are shown in the fluorescence photomicrographs in Fig. 5. Normal cell nuclei are stained red by propidium iodide; fragmented DNA typical of apoptotic cell nuclei additionally incorporates the green fluorescein label. The combination results in the appearance of yellow fluorescent apoptotic cell nuclei.

Results of cell death ELISA assay of control and dust-treated cells are shown in Fig. 6. Cell challenge by native quartz resulted in a dose-dependent increase in cytosolic histone-bound DNA fragments, measured by optical density. Native kaolin induced significant response at the two highest concentrations. At the highest native dust concentration, the native quartz expressed about two and a half times the activity of the native kaolin.

Cells also were challenged over a 5-day period at a concentration of 100 μg/ml of native or DPPC-treated quartz or kaolin and assayed at 6 and 12 h and 1, 3, and 5 days by the cell death ELISA assay, with results shown in Fig. 7. Native quartz induced a time-dependent increase in apoptosis over a 12-h period, which then decreased gradually with exposure time between 1 and 5 days, while remaining significant at $p < 0.01$ at all times. Challenge with native kaolin resulted in no activity at 6 h through 3 days, with activity significant at $p < 0.01$ expressed at 5 days. DPPC pretreatment of quartz suppressed apoptotic activity to background levels over a 1-day period, but this activity was restored and was significant at $p < 0.01$ at 3 and 5 days after cell challenge. No apoptotic activity was found in cells challenged with DPPC-treated kaolin.

To further confirm the results of morphology, TUNEL and cell death ELISA assays for apoptotic response, a DNA agarose gel study tested for the presence of internucleosomal DNA fragmentation, which is a characteristic feature of apoptotic cells. As shown in Fig. 8, treatment of NR8383 with native quartz for 6 h at concentrations from 50 to 400 μg/ml resulted in readily seen DNA ladder formation; there appeared to be a qualitative dust concentration-dependent increase in intensity of the ladder bands. Native kaolin at concentrations from 50 to 400 μg/ml did not appear to result in DNA ladder formation qualitatively discernable above controls. Figure 9 shows the result of DNA ladder assays of cells after treatment over a 5-day period at a concentration of 100 μg/ml of untreated and DPPC-treated quartz dust. Native quartz resulted in visible DNA ladder formation at all time points; the activity appeared to decrease with time after the 12-h point. DPPC pretreatment of quartz suppressed its DNA ladder formation over a 1-day period, but this DNA ladder formation was clearly visible at 3 and 5 days after challenge. No DNA ladder formation was apparent for cells treated with native and DPPC-treated kaolin at dust concentration of 100 μg/ml for a 5-day period.

DISCUSSION

Both native quartz and native kaolin caused a concentration- and time-dependent increase in cytotoxicity to the NR8382 rat alveolar macrophage cell line as measured by an *in vitro* LDH release assay. Native quartz dust challenge caused a statistically significant increase in cell-released LDH activity above background at all native quartz dust concentrations; and LDH activity increased with increasing dust concentration. In contrast, kaolin cytotoxic activity was not seen at the two lowest kaolin concentrations. Some previous short-term *in vitro* cytotoxicity and membranolysis assays found comparable activities for these quartz and kaolin dusts, such as seen at the highest concentration in this study (Vallyathan *et al.*, 1988; Wallace *et al.*, 1985, 1988, 1992). For example, a 2-h incubation of native quartz and kaolin with primary lavaged rat alveolar macrophages in serum-free buffer resulted in slightly greater LDH release induced by the kaolin at equal mass concentrations of 1000 μg/ml (Wallace *et al.*, 1985). And *in vitro* erythrocyte hemolysis studies in serum-free medium have found this stock of kaolin to express comparable and somewhat greater membranolytic activity than this stock of quartz, with the activity of both dusts fully suppressed by DPPC adsorption adequate to provide a bilayer lipid coating on the dust surfaces (Wallace *et al.*, 1992). However, in another *in vitro* cytotoxicity experiment with these dusts using serum-containing medium, exposure of lavaged rat pulmonary macrophages at quartz concentrations up to 6.7 μg/ml and kaolin up to 13.4 μg/ml indicated native quartz to be almost twice as active as native kaolin in a "live–dead" fluorescence assay for cell viability (Gao *et al.*, 2000). The low cytotoxicity seen at low native kaolin concentrations in the current study may reflect an innate mineral-specific difference in native dust cytotoxicity between quartz and kaolin seen only at the lower dust concentrations. However, another possible cause is a mineral-specific passivation effect in this *in vitro* system, e.g., prophylactic components of serum in limited amounts may preferentially deactivate kaolin, until higher kaolin concentrations deplete the components. Earlier short-term *in vitro* studies showing comparable cytotoxic activities of quartz and kaolin were performed using serum-free medium, while the current study and the prior study of Gao *et al.* (2000) showing diminished kaolin activity used serum-containing medium. The prophylactic effects of some protein components of serum have been demonstrated for cristobalite silica dust (Barrett *et al.*, 1999). Studies of silica adsorption of some endogenous proteins have been reviewed (Fubini and Wallace, 2000). And kaolin is recognized to be an effective sorbent for many compounds, including proteins from aqueous solution.

The TUNEL and cell death ELISA assays found that, similar to their necrotic activities, quartz caused a monotonic concentration-dependent increase in apoptosis of NR8383 cells *in vitro*, while kaolin was active only at the highest concentra-

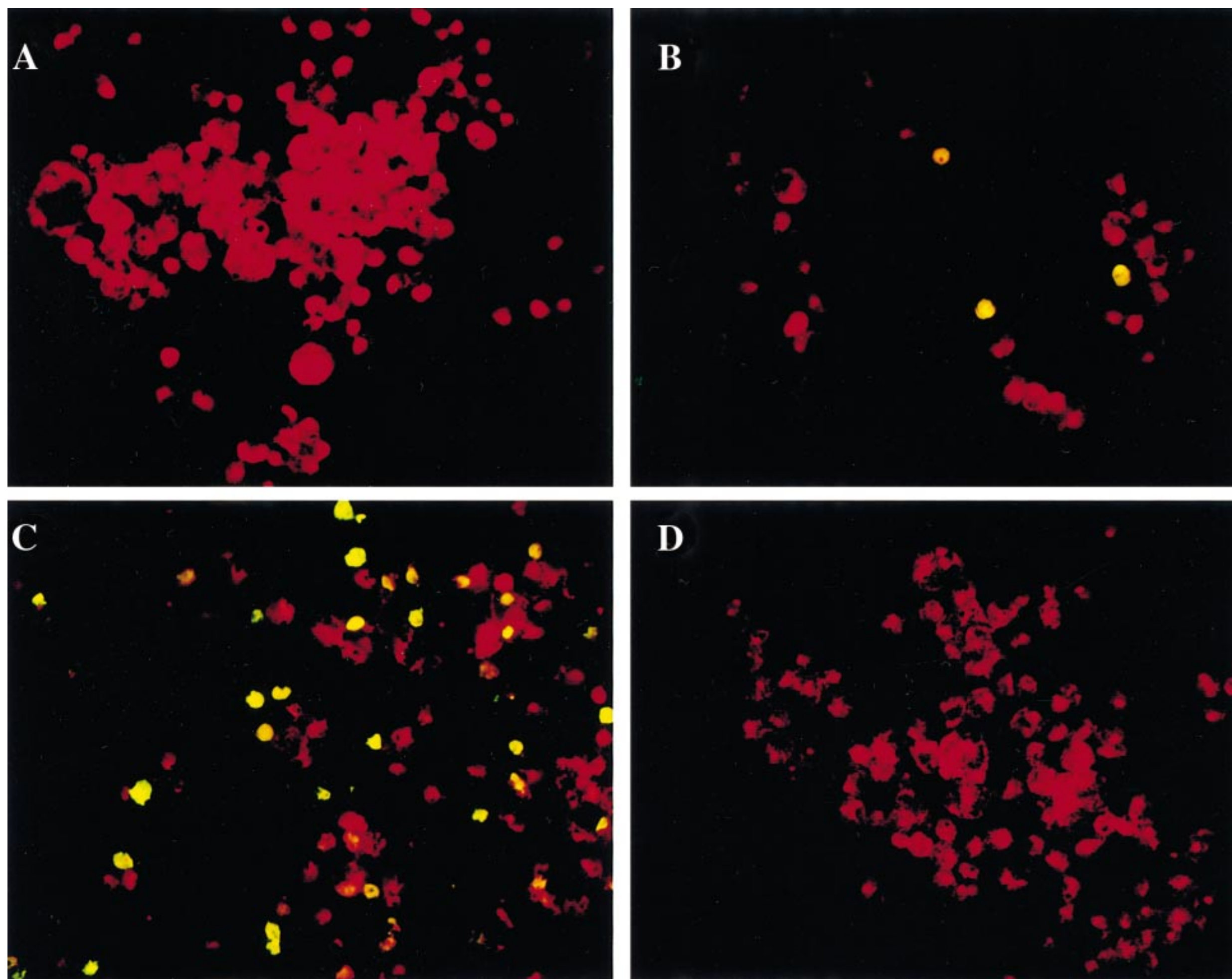

FIG. 5. Fluorescence microscopy appearance of NR8383 rat alveolar macrophages stained by the TUNEL technique on treatment with quartz or kaolin: (A) control, (B) 50 μg/ml quartz, (C) 400 μg/ml quartz, and (D) 200 μg/m kaolin. Cells were challenged with quartz or kaolin for 6 h. Quartz caused apoptosis in rat AM at 50 to 400 μg/ml, whereas kaolin did not induce apoptosis at 50 to 200 μg/ml and caused limited apoptosis at 400 μg/ml. Cells were double-stained with fluorescein-12-dUTP and propidium iodide. Normal cells show red nuclei, whereas apoptotic cells show yellow nuclei. Magnification 1000× (oil).

tions. Native quartz was about twice as active in the apoptosis assays as native kaolin at the highest dust concentration. There is a comparable pattern of behavior between the necrosis and apoptosis activities with dust concentration, i.e., quartz shows a monotonic increase in both activities with concentration while kaolin activities were significantly expressed only at the higher dust concentrations; and kaolin activities were not as strong as the activities of an equal mass concentration of quartz dust. Native quartz at the intermediate concentration (100 μg/ml) induced a nonlinear time-dependent increase in apoptosis, increasing for 12 h and then decreasing but remaining significant at 5 days as measured by the cell death ELISA and DNA ladder formation assays. That decrease may be the result of the increase in cytotoxic cell death with a subsequent decrease in cells available for apoptotic response. Native kaolin at that concentration did not express significant apoptotic activity until the 5-day time point. Quartz is known to induce cell apoptosis *in vitro*: Sarih *et al.* (1993) found that silica-treated macrophages released high amounts of IL-1β and underwent apoptosis. Several other agents are known to be both fibrogenic and apoptotic: bleomycin, a fibrogenic agent, has been reported to cause alveolar macrophage apoptosis (Hamilton *et al.*, 1995). Chrysotile and crocidolite asbestos, fibrogenic particulates, have been reported to cause apoptosis, whereas wollastonite, a nonfibrogenic fiber, does not induce apoptosis (Hamilton *et al.*, 1996). Hogquist *et al.* (1991) have also demonstrated a possible relationship between IL-1β secretion and cell apoptosis. Recent studies have provided evidence that certain cytokines such as tumor necrosis factor-α (TNF-α) and transforming growth factor beta (TGF-β) play a key role in silica-induced inflammation and fibrosis (Piguet *et al.*, 1990; Williams *et al.*, 1993). TNF-α and TGF-β have been shown to induce apoptosis in different types of cells (Pierce *et al.*, 1991; Sarin *et al.*, 1995; Bursch *et al.*, 1993; Tsuchida *et al.*, 1995).

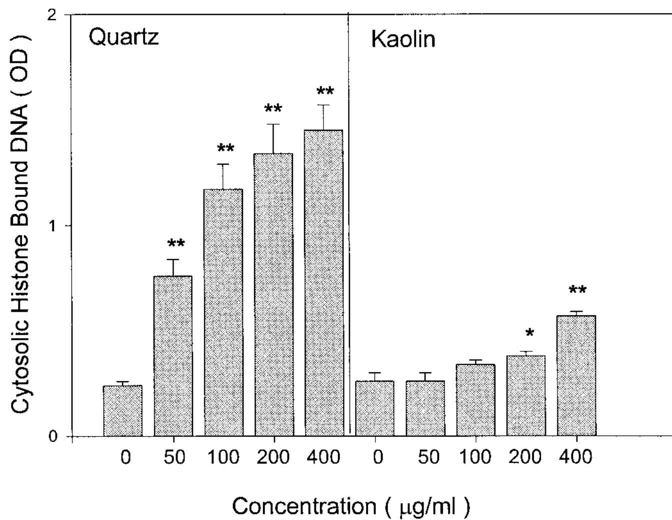

FIG. 6. Effects of quartz and kaolin on apoptosis in NR8383 rat pulmonary macrophage after challenge for 6 h using cell death ELISA assay. Values represent the mean cytosolic histone-bound DNA (OD) ± SE for five replications of this experiment. Statistical significance comparing quartz or kaolin to control as determined by Dunnett's t test at $*p < 0.05$, $**p < 0.01$.

However, the nature of a cause and effect relationship between the apoptotic potential of dusts or fibers and their ability to produce fibrosis is not certain. In the current study, the parallel behavior of cytotoxic and apoptotic activities with dust concentration and with time of cell challenge suggests but does not prove that the apoptotic activity may be a response of some cells in the culture to cytokines or other factors released by nearby cells undergoing necrotic response to the dust.

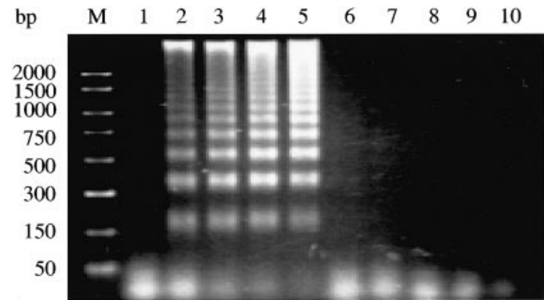

FIG. 8. Agarose gel electrophoresis of DNA extracted from control, quartz-, or kaolin-treated NR8383 rat alveolar macrophages. Sequential DNA fragmentation resulting in banding or ladder appearance is characteristic of apoptosis. Cells were challenged with quartz or kaolin for 6 h. Quartz caused DNA ladder formation in rat AM at 50 to 400 μg/ml, whereas kaolin did not. Electrophotograms from left: lane M, commercial DNA size marker; lanes 1 and 6, control; lanes 2–5, 50, 100, 200, and 400 μg/ml of quartz; lanes 7–10, 50, 100, 200, and 400 μg/ml of kaolin.

DPPC surfactant pretreatment of quartz dust fully suppressed its cytotoxicity and apoptotic activity at the early time points. Apoptotic and necrotic activities expressed by native quartz at 6 and 12 h and 1-day time points were eliminated. DPPC surfactant treatment of kaolin eliminated the apoptotic activity that had begun to be expressed by the native kaolin at 5 days. Restoration of quartz activities at 3 and 5 days is consistent with other studies of the *in vitro* rates of surfactant digestion from quartz particles by digestion processes in some other cell lines: In cell-free systems, phospholipase enzymes can remove adsorbed DPPC surfactant from quartz and kaolin dusts and restore dust toxicity that had been suppressed by

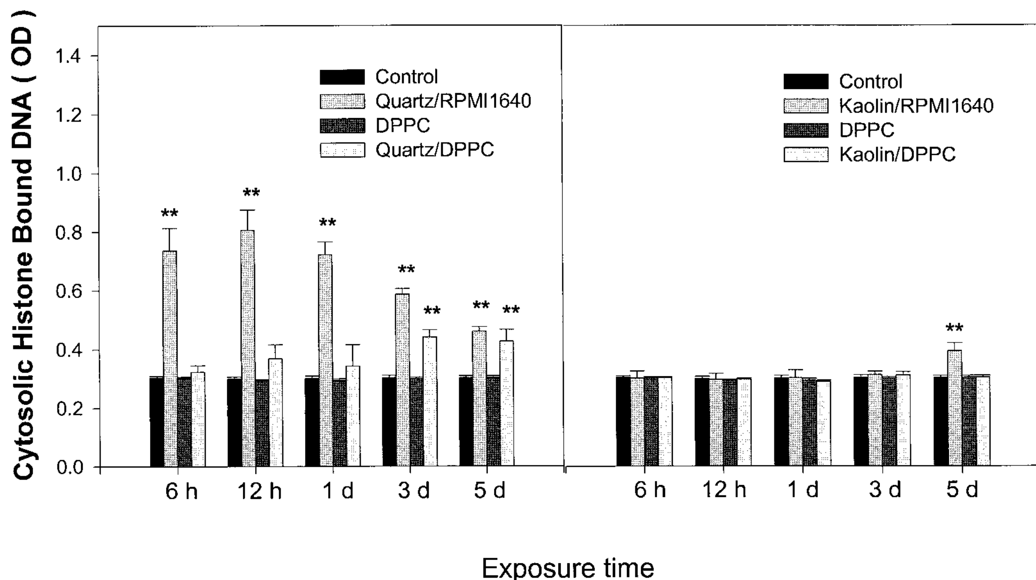

FIG. 7. Time course of untreated and DPPC-treated quartz and kaolin induction of apoptosis in NR8383 rat pulmonary macrophages after exposure of 100 μg/ml of dusts using cell death ELISA assay. Values represent the mean cytosolic histone-bound DNA (OD) ± SE for five replications of this experiment. **Statistical significance comparing untreated or DPPC-treated quartz to control as determined by Dunnett's t test at $p < 0.01$.

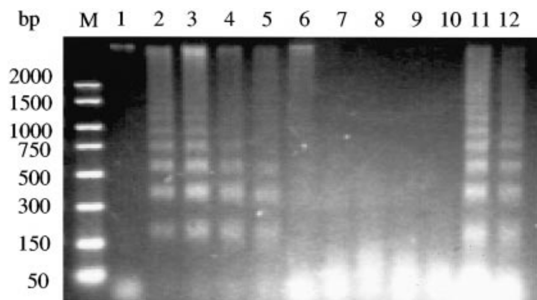

FIG. 9. Agarose gel electrophoresis of DNA extracted from control, quartz-treated, and quartz/DPPC-treated NR8383 rat alveolar macrophages. Sequential DNA fragmentation resulting in banding or ladder appearance is characteristic of apoptosis. Cells were challenged with quartz at 100 μg/ml for a 5-day period. Electrophotograms from left: lane M, commercial DNA size marker; lanes 1 and 7, control; lanes 2–6, 6 h, 12 h, 1 day, 3 days, and 5 days after cell exposure to quartz; lanes 8–12, 6 h, 12 h, 1 day, 3 days, and 5 days after cell exposure to DPPC-treated quartz.

DPPC adsorption (Wallace *et al.*, 1988, 1992). Cellular response to DPPC-treated quartz was observed over a 3-day period for macrophages *in vitro* (Antonini *et al.*, 1994). *In vitro* digestion of DPPC from quartz and kaolin by the macrophage P388D1 cell line was measured over a 9-day period (Hill *et al.*, 1995). A parallel time course has been seen for the *in vitro* removal of DPPC surfactant and restoration of quartz toxicity to macrophages over a 7-day period (Liu *et al.*, 1998). The current study results are consistent with the theory that phospholipid surfactant adsorption onto mineral dust surfaces suppresses their prompt toxicities and that digestive removal of this coating is associated with their restoration.

In summary, the current study demonstrates distinctive *in vitro* necrotic and apoptosis behavior between quartz, a strongly fibrogenic dust, and kaolin, a dust with relatively weak pathogenic potential. Quartz induced necrotic and apoptotic activity at all concentrations tested, while kaolin expressed activity only at the higher concentrations. For a given mineral, the time-course and dose–response behavior for its cytotoxic activity and its apoptotic activity were similar, suggesting necrosis of some cells may have induced apoptotic activity in others. DPPC surfactant pretreatment of the dusts had a transient prophylactic effect on their activities. Kaolin dust cytotoxic activity seen in this study is weak compared to that of quartz; this is in contrast to results of some previous studies in which quartz and kaolin were comparably cytotoxic *in vitro* in serum-free medium. This suggests additional prophylactic effects, e.g., of components of the serum medium, may have preferentially affected the kaolin activities at low dust concentrations under conditions used in the current study.

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