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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · OCTOBER 2002

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Chem. Res. Toxicol., **2002**, 15 (9), 1166-1173• DOI: 10.1021/tx025558u • Publication Date (Web): 24 August 2002

Surface Modification of Quartz Inhibits Toxicity, Particle Uptake, and Oxidative DNA Damage in Human Lung Epithelial Cells

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Received May 14, 2002

Quartz (crystalline silica) is not consistently carcinogenic across different industries where similar quartz exposure occurs. In addition, there are reports that surface modification of quartz affects its cytotoxicity, inflammogenicity, and fibrogenicity. Taken together, these data suggest that the carcinogenicity of quartz is also related to particle surface characteristics, and so we determined the genotoxic effects of DQ12 quartz particles versus DQ12 whose surface was modified by treating with either aluminum lactate or polyvinylpyridine-*N*-oxide (PVNO). The different particle preparations were characterized for hydroxyl-radical generation using electron spin resonance (ESR). DNA damage was determined by immunocytochemical analysis of 8-hydroxydeoxyguanosine (8-OHdG) and the alkaline comet-assay using A549 human lung epithelial cells. Cytotoxicity was measured using the LDH- and MTT-assays, and particle uptake by the A549 cells was quantified by light microscopy, using digital light imaging evaluation of 800 nm sections. The ability of quartz to generate hydroxyl-radicals in the presence of hydrogen peroxide was markedly reduced upon surface modification with aluminum lactate or PVNO. DNA strand breakage and 8-OHdG formation, as produced by quartz at nontoxic concentrations, could be completely prevented by both coating materials. Particle uptake into A549 cells appeared to be significantly inhibited by the PVNO-coating, and to a lesser extent by the aluminum-lactate coating. Our data demonstrate that respirable quartz particles induce oxidative DNA damage in human lung epithelial cells and indicates that surface properties of the quartz as well as particle uptake by these target cells are important in the cytotoxic and the genotoxic effects of quartz in vitro.

Introduction

Silicosis has been recognized for many decades as one of the most prevalent occupational lung diseases in the world (*1*, *2*). More recently, respirable quartz has also been classified as a carcinogen by IARC/WHO (*3*). The carcinogenicity of quartz has been mainly inferred from a number of chronic animal studies and epidemiological studies, as reviewed by IARC (*3*). Nevertheless, the exact mechanisms by which quartz exposure may cause lung cancer are not yet fully understood (*2*, *4*). Surface properties of quartz play a crucial role in its ability to form ROS,[1] as demonstrated in ESR studies of quartz using various surface modifying methods, such as grinding, etching or coating (*5*−*9*). Since ROS are implicated in DNA damage and carcinogenesis (*10*−*12*), these intrinsic surface properties of quartz may therefore play a role in its carcinogenesis. Support for the involvement of ROS in the DNA-damaging properties of quartz comes from animal studies showing enhanced formation of the hydroxyl radical specific DNA lesion 8-OHdG in lungs of rats intratracheally instilled with quartz (*13*−*15*). Although the excessive and persistent formation of ROS by activated neutrophils and macrophages is considered to be a crucial factor in these effects (*16*−*19*), direct effects of particles cannot be fully excluded in such in vivo studies (*15*, *20*, *21*). Studies with quartz using naked DNA as a target suggest that ROS formation by the particle itself may indeed play a role in the genotoxicity of quartz (*22*, *23*), and in line with this, we and others have demonstrated in vitro genotoxicity of quartz particles (*24*−*29*).

Asbestos is another mineral dust carcinogen, and the significance of ROS formation in its genotoxic properties is supported from a number of recent studies, demonstrating that these fibers cause formation of 8-OHdG in vitro (*30*−*33*). However, recent data also emphasize that these DNA-damaging effects depend on phagocytosis of these fibers by the target cells, and intracellular oxidative stress (*34*, *35*). For quartz however, it is less well-

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[1] Abbreviations: ROS, reactive oxygen species; PVNO, polyvinylpyridine-*N*-oxide; 8-OHdG, 8-hydroxydeoxyguanosine; ESR, electron spin resonance; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,-diphenyltetrazolium bromide; ICP-MS, inductively coupled plasma mass spectrometry; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide.

10.1021/tx025558u CCC: $22.00
Published on Web 08/24/2002

established whether these intrinsic physicochemical properties of the particles are important for the initiation of DNA damage in target cells.

In the overall evaluation of quartz by IARC, the working group emphasized that its carcinogenicity may depend on "inherent characteristics of the material" (*3*), i.e., that quartz may not behave as a carcinogen under all circumstances (*4*, *9*). For risk assessment purposes, this statement necessitates thorough investigation of the properties of quartz that determine its possible carcinogenicity. In the past decades, physicochemical properties of quartz particles have been related to cytotoxicity and fibrogenicity (*2*, *4*, *5*, *9*). Several research groups have for instance demonstrated that the toxic effects of quartz may be reduced if the surface is modified. For instance, haemolytic activity of quartz can be inhibited upon coating with PVNO (*36*, *37*) or with organosilane (*6*). Coating with aluminum lactate has been reported to reduce cytotoxicity of quartz (*38*) and has been demonstrated to result in a reduction of pulmonary inflammation when instilled into the lungs of rats or sheep (*39*−*42*). However, it has not been investigated whether these coating modifications can affect the DNA-damaging properties of quartz particles.

The aim of our present study was to determine the effects of coating of respirable quartz particles on the induction on DNA damage in lung epithelial cells and to investigate whether ROS generation by the particles and phagocytosis play a role in the observed effects. Therefore, we studied the effect of either aluminum lactate or PVNO coating on quartz-induced DNA damage, in A549 human lung epithelial cells, using the comet-assay and immunocytochemical analysis of 8-OHdG. The genotoxic effects of these different particle treatments were related to their hydroxyl-radical generation capacities, as well as to cytotoxicity and particle uptake in the epithelial cells. In the present study we demonstrate, in lung epithelial cells, that aluminum lactate and PVNO coating reduces the genotoxic activity of quartz and that, in the absence of cytotoxicity, radical generation properties as well as particle uptake play an important role in the induction of DNA damage by respirable quartz particles.

Materials and Methods

Surface Modification of Quartz Particles. For all experiments, DQ12 quartz (Batch 6, IUF, Düsseldorf) particles were used. To modify the surface properties, the quartz particles were treated with either aluminum lactate (Sigma-Aldrich, Taufkirchen, Germany) or PVNO (*43*). The quartz was suspended at a concentration of 5 mg/mL in 1% solutions of either aluminum lactate or PVNO in distilled water, subsequently sonicated for 5 min, and agitated for 3 h at room temperature. Quartz suspended in distilled water, sonicated and agitated for the same time intervals, was used as a control. The protocol was based on procedures as described earlier for both compounds (*44*, *45*), with some mutual modifications, so only one sham-coating procedure would be required. Following agitation, each suspension was washed twice by centrifugation (5 min, 13 000 rpm) in phenol red-free Hanks Balanced Salt Solution and immediately used for experiments. To determine whether surface modification with aluminum lactate or PVNO caused changes in particle size distribution or aggregation of the DQ12, transmission electron microscopy was used. No differences were found in the size distribution of the different quartz preparations (*42*). The efficacy of the aluminum lactate coating procedure was investigated by atomic absorption spectrometry analysis following 3 h treatment of the coated quartz in 1 M HNO_3 at 80 °C. The coating efficacy of PVNO was measured by spectrophotometric determination at 240 nm (Beckman) of the desorbed PVNO upon 3 h treatment in 1 M NaOH at 80 °C. The estimated adsorbed amounts on the quartz were found to be 11 μg of PVNO/mg of quartz (3.5 mg/m^2 quartz) and 4 μg of aluminum/mg quartz (1.3 mg/m^2 quartz, or 48.2 μmol/m^2 quartz).

Electron Spin Resonance. The ability of the (surface modified) quartz to hydroxyl-radical release was evaluated by ESR as described elsewhere (*46*, *47*) with some minor modifications. Briefly, 250 μL of particle suspension was mixed with 250 μL of 0.5 M H_2O_2 in phosphate buffered saline (PBS) and 500 μL of 0.05M of DMPO (Sigma-Aldrich, Taufkirchen, Germany) in distilled deionized water. The suspension was incubated for 15 min at 37 °C, and filtered through a 0.22 μm pore filter (Sartorius, Göttingen, Germany). The clear filtrate was then transferred to a capillary and spin trap signal was measured with a Miniscope ESR spectrometer (Magnettech, Berlin, Germany). The ESR spectra were recorded at room temperature using the following instrumental conditions: magnetic field, 3360 G; sweep width, 100 G; scan time, 30 s; number of scans, 3; modulation amplitude, 1.975 G; receiver gain, 1000. Quantification was done by accumulation of three different spectra, each averaging three different scans. The spectra were quantified by double integration. As a positive particle control a coalfly ash was used (MAT-41) as described previously (*46*). As a negative control, a mixture of water, H_2O_2, and DMPO was used. The ESR analyses for the different quartz preparations was performed in three independent experiments, each representing different freshly prepared suspensions that were incubated and analyzed in duplicate.

Culture of A549 Cells. A549 cells (American Type Culture Collection), were grown in DMEM culture medium, supplemented with 10% heat inactivated foetal calf serum (Sigma-Aldrich, Taufkirchen, Germany), L-glutamine, and 30 IU/mL penicillin-streptomycin at 37 °C and 5% CO_2. Experiments were performed in serum-free DMEM. Although the A549 cells are transformed, they have structural and biochemical characteristics of human type II cells and are known to ingest particles (*48*). Furthermore, A549 cells have been successfully applied by several research groups to investigate the occurrence of DNA damage by, e.g., asbestos fibers or ambient particulate matter (*30*, *33*, *49*).

Cytotoxicity. Cytotoxicity was determined using two different assays, i.e., the MTT-assay as an indicator of the metabolic competence of the cells, and the LDH assay to determine plasma membrane leakage. The MTT-assay was performed as described by Mosmann et al. (*50*), with some minor modifications. Briefly, cells were seeded at 15 000 cells/200 μL into 96-well flat-bottom culture plates, grown for a further 48 h and then treated with the particle suspensions at the indicated concentrations and time intervals. At the end of each incubation, 25 μL of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, Taufkirchen, Germany) in PBS was added for a further 3 h. The medium was then discarded, 200 μL of DMSO was added to each well, and the plate was agitated for 1 min. Absorption at 450 nm was quantified with a microplate reader (Multiskan Ascent, Labsystems, Frankfurt, Germany). MTT-reduction for each treatment was expressed as a percentage of control values. The LDH-assay was based on the method as described elsewhere (*51*). Briefly, lactate dehydrogenase (LDH) activity was measured at 540 nm using pyruvic acid as the substrate. LDH release into the medium was determined as a percentage of total cellular LDH. Total cellular LDH was measured in cell lysates obtained by treatment with 0.1% Triton X-100.

Single Cell Gel Electrophoresis. DNA strand break formation was determined by the comet-assay (*52*−*54*), as follows. Cells were seeded into 60 mm diameter culture dishes (5×10^5 cells/dish) and grown for a further 48 h. On the day before the experiments, fully frosted slides were covered with a layer of 0.65% agarose using a coverslip and stored overnight at 4 °C.

Following treatment of the cells with (coated) quartz for 4 h, the monolayers were rinsed twice with PBS. Cells were detached with trypsin-EDTA (500 μL, 2 min), and immediately suspended in complete culture medium. Cells were centrifuged for 5 min at 800*g*, and resuspended in PBS. Cell yield and viability of each incubation was assessed by a Neubauer hematocytometer using trypan blue dye exclusion. Slides (covered the day before with agarose) were then covered with a second layer containing a mixture of 25 μL of cell suspension and 75 μL of 0.65% low melting point agarose. The slides were stored for 1 h at 4 °C to allow solidification and then covered with another layer of the low melting point agarose. Following solidification for at least 1 h at 4 °C, slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, pH 10; 10% DMSO and 1% Triton X-100 added just before use) and stored overnight at 4 °C. The following day, slides were rinsed with distilled water and then placed in an electrophoresis tank filled with ice-cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min. The electrophoresis tank was kept in an ice bath. Electrophoresis was conducted at 300 mA and 25 V for 15 min. Slides were then neutralized three times for 5 min using neutralization buffer (0.4 M Tris, pH 7.5). All steps described were performed in the dark or under dimmed red light to prevent additional DNA damage. Slides were stained with ethidium bromide (2 μg/mL in H_2O) and comet appearance were analyzed on an Olympus BX60 fluorescence microscope at 1000× magnification. For each individual experiment, per treatment at least 2 × 25 cells were analyzed randomly and classified into one out of five categories according to tail length (I, II, III, IV, and V, in which I = undamaged cells without comet tail) (*54*).

Measurement of 8-Hydroxydeoxyguanosine. The OH-radical specific DNA lesion, 8-OHdG, was measured by immunocytochemistry as described previously (*47*), with some modifications. Briefly, cells were seeded in four-chamber slides (Falcon, Becton Dickinson, Meylan, France) at a concentration of 120 000 cells/chamber. After 2 days, cells were exposed to (coated) quartz for 4 h. Immunocytochemistry was performed using the Vectastain-ABC kit (Vector Laboratories,Burlingame, CA) and using the N45.1 monoclonal antibody against 8-OHdG (*55*). The 8-OHdG was quantified using digital imaging analysis system software (SIS, Münster, Germany). Of each individual sample, the total staining intensity was measured and divided by the number of counted cells, counting at least 200 cells/experiment. The relative staining intensity of 8-OHdG was expressed as a percentage of control.

Particle Uptake. Particle uptake in A549 cells was determined as follows. Cells cultured in 60-mm diameter tissue culture dishes as described above were treated with (coated) particles for 2, 4, and 24 h. At the end of each incubation, the monolayers were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at 4 °C. After postfixation in 2% OsO4, samples were stained with 1.5% uranyl acetate dihydrate and tungstphosphoric acidhydrate, dehydrated in ethanol series and embedded in Epon (Serva, Heidelberg, Germany). Sections of 800 nm, two blocks per sample, were then stained with azurII-methylenblue (Richardson) and analyzed by light microscopy at 1000× magnification. The method applied by us here allows analysis of the intracellular localization of particles with preservation of the native organization and polarity of the monolayer cultures. For each section, at least 200 cells were counted interactively, using digital imaging (SIS, Münster) at 1500× magnification. For each individual cell the number of ingested particles was determined. Separate sections were also prepared for transmission electron microscopy analysis (Philips CM12).

Statistical Analysis. Data are expressed as mean ± standard deviations unless stated otherwise. Statistical analysis was performed using SPSS v.10 for Windows. Treatment-related differences were evaluated using Student's *t*-test or using one-way ANOVA. Multiple comparisons were then evaluated using Tukey's method. For analysis of the comet data, the distribution of cell damage classifications was taken into account using the χ^2-test. A difference was considered significant at $p < 0.05$.

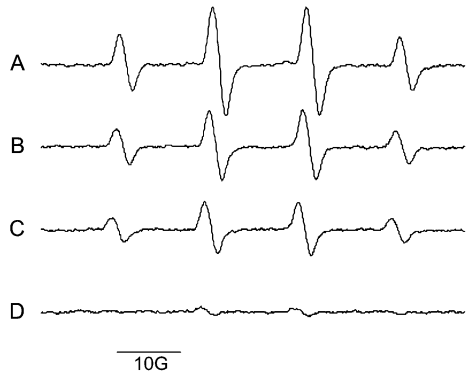

Figure 1. Hydroxyl-radical generation of the different quartz preparations. The different quartz preparations were incubated for 15 min in the presence of DMPO and hydrogen peroxide. A = Quartz; B = Quartz treated with aluminum lactate; C = quartz treated with PVNO; D = control (hydrogen peroxide without quartz).

Results

Hydroxyl-Radical Formation by Quartz and Surface-Coated Quartz. Electron spin resonance was used to determine the hydroxyl-radical generation properties of the DQ12 quartz particles following different treatments. Representative spectra are shown in Figure 1. For all particle preparations, a clear 1:2:2:1 quartet pattern was shown with a split centre at 3400 G, characteristic for the spectrum of [DMPO-OH]•(*5*). Modification of the quartz surface with aluminum lactate or PVNO was found to cause a significant ($n = 3$, $p < 0.05$) reduction of the formation of [DMPO-OH]• adducts. The quantified signals (arbitrary units) were 2.94 ± 0.55 for the control quartz, 1.88 ± 0.35 for the PVNO-treated quartz, and 1.64 ± 0.65 for the aluminum lactate treated quartz, respectively.

Cytotoxicity of Quartz and Coated Quartz Particles. The cytotoxic effects of DQ12 particles with or without coating on the A549 cells was studied by two independent assays, i.e., the MTT-assay and the LDH-assay. The results of the MTT-assay, as a measure of metabolic competence of the cells following 4 h treatment with the different quartz preparations, are shown in Figure 2. The toxicity of quartz, observed at a concentration of 200 μg/cm^2, was significantly inhibited upon coating with aluminum lactate or PVNO. Inhibition of the toxicity of the quartz seemed to be more effective for the PVNO-coated quartz particles. The effects of quartz and coated quartz particles on cell membrane integrity, as determined by the LDH assay, are shown in Figure 3. None of the particle treatments caused a significant membranolytic effect at 4 h, with the exception of quartz at the highest concentration, i.e., 200 μg/cm^2. Following 24 h incubation, significant toxicity was observed with quartz, and with the aluminum-lactate-coated quartz at the highest concentration, i.e., 200 μg/cm^2. At this time point, again the strongest inhibition of toxicity appeared to occur with the PVNO coating.

Formation of DNA Strand Breaks by Quartz and Coated Quartz. DNA strand break formation by quartz particles was determined by the comet-assay. Earlier, we showed that DQ12 causes a dose-dependent increase in the formation of DNA strand breaks in A549 cells (*29*).

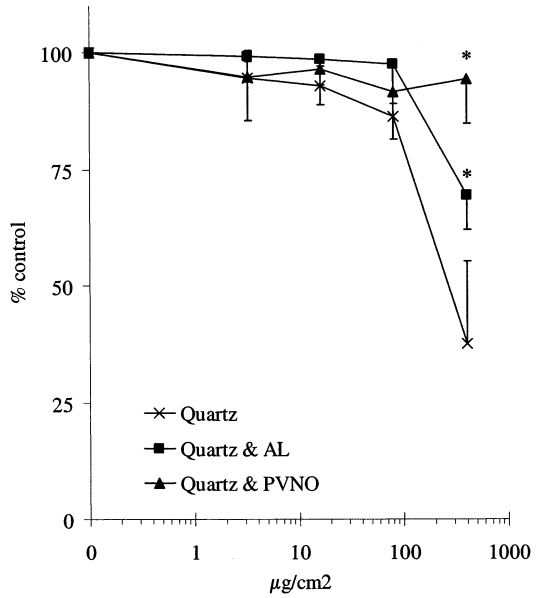

Figure 2. Cytotoxicity (MTT-assay) of the different quartz preparations in A549 human lung epithelial cells. Cells were treated for 4 h with quartz particles with or without surface modification with aluminum lactate or PVNO at the indicated concentrations. Cytotoxicity was measured using the MTT-assay as a measure of metabolic competence of the cells. The viability of the cells for each treatment and concentration is expressed as a percentage of control values. Data are expressed as mean and standard deviations of three independent experiments, each using 5 replicate wells per concentration. (*) $p < 0.05$ vs Quartz.

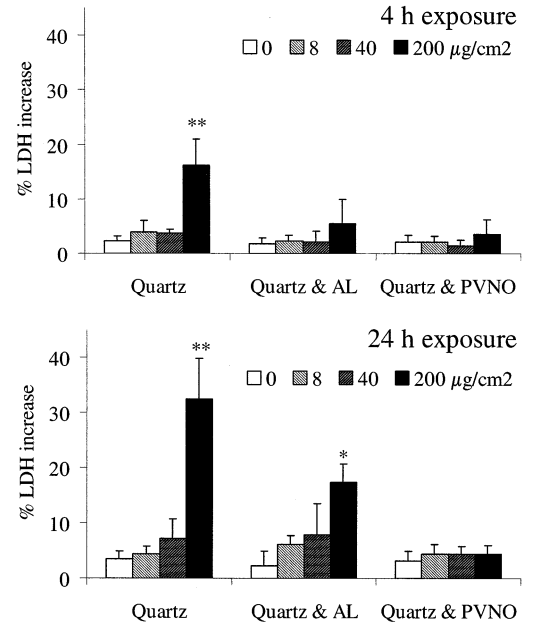

Figure 3. Cytotoxicity (LDH-assay) of the different quartz preparations in A549 cells. Cells were treated for 4 (top panel) or 24 h (bottom panel) with quartz particles with or without surface modification at the indicated concentrations. Cytotoxicity was determined using the LDH assay as an indicator of cell membrane damage. Data are expressed as the percentage of extracellular LDH and represent mean and standard deviations of three independent experiments, each using duplicate wells per concentration. (*) $p < 0.05$ and (**) $p < 0.01$ versus control.

To study the effects of quartz coatings in the comet-assay, cells were treated with the different particle preparations for 4 h at a concentration of 40 μg/cm^2, chosen on the basis of absence of toxicity in the MTT-assay as well as in the LDH-assay. The effects of coating on DNA strand break formation induced by the quartz is shown in Figure 4. The formation of strand breaks in A549 cells by quartz was significantly reduced for the coated quartz particles. Parallel analysis of the cells used for the comet assay, using trypan blue dye exclusion, further confirmed that the observed effects were not due to cytotoxic effects during incubation nor due to subsequent processing for the comet-assay.

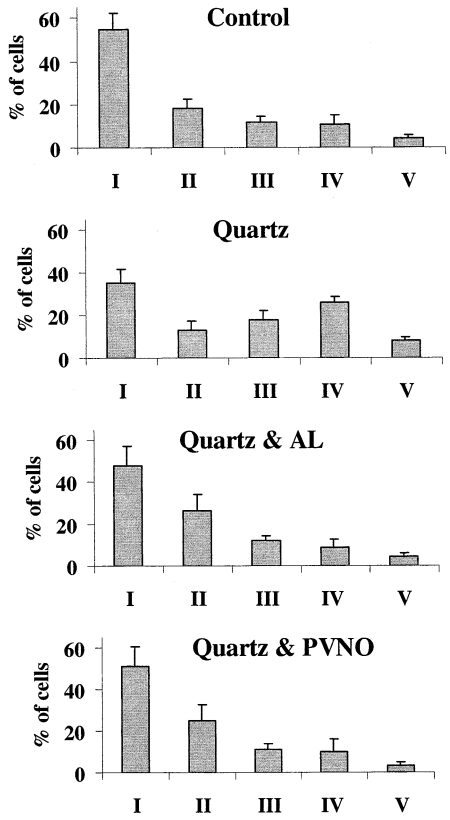

Figure 4. DNA strand break formation by quartz with or without coating. A549 cells were treated for 4 h with quartz with or without surface modification at a concentration of 40 μg/cm^2. Data are shown as mean and standard error of comet classes according to comet tail length as determined for three independent experiments, using two slides per treatment (see methods section for details). DNA strand breakage observed in the cells treated with quartz was significantly higher (χ^2, $p < 0.01$) compared to untreated cells (controls), as well as to DNA damage as observed in the cells treated with the modified quartzes (aluminum lactate, PVNO).

Formation of 8-OHdG by Quartz and Coated Quartz. The formation of oxidative DNA damage by DQ12 was determined using immunocytochemistry analysis of 8-OHdG. At a concentration of 80 μg/cm^2, DQ12 particles were found to induce 8-OHdG in A549 cells. The effects of coating on 8-OHdG staining are shown in Figure 5, which illustrate that oxidative DNA damage was increased only by non-coated quartz particles compared to controls. The staining intensity in the quartz treated cells as determined by digital imaging quantification (see methods section for details) was 210 ± 17% of control. However, in the quartz particles that had been coated with either aluminum lactate or PVNO 8-OHdG was not different from controls (see Figure 5).

Cellular Uptake of DQ12 Particles with or without Surface Coatings. The effects of the different coatings on the intracellular uptake of the quartz in A549 cells are shown in Table 1. After 2 h of exposure, most of the epithelial cells had already ingested quartz particles,

Table 1. Uptake of Quartz and Coated Quartz Particles in A549 Cells[a]

time	quartz	quartz and aluminum	quartz and PVNO
% of cells with particles			
2 h	93.7 (91.7−94.6)	69.9 (57.6−86.4)[c]	11.8 (10.0−13.7)[c,d]
4 h	88.3 (87.7−88.9)	45.2 (17.5−81.4)	14.3 (4.4−27.2)[c]
24 h	79.7 (78.7−80.6)	84.4 (83.7−85.1)	10.1 (6.1−12.1)[c,d]
no. of particles/ϕc cell[b]			
2 h	7.0 (6.7−7.4)	3.6 (2.2−4.4)[c]	1.7 (1.3−2.1)[c,d]
4 h	5.2 (4.4−5.9)	3.2 (2.0−4.1)[c]	2.4 (1.5−3.5)[c]
24 h	5.3 (4.2−6.6)	5.6 (5.5−5.6)	1.9 (1.5−2.3)[c,d]

[a] Data are expressed as a mean and, between parentheses, range. [b] Number of particles per phagocytosing cell, i.e., as determined for those cells that contained at least one particle. [c] $p < 0.05$ vs quartz. [d] $p < 0.05$ vs quartz and aluminum.

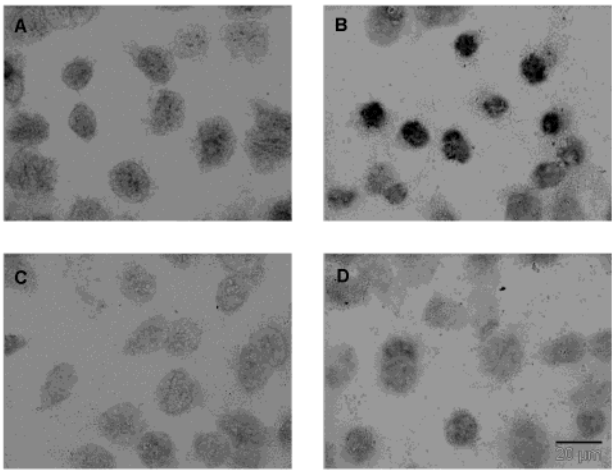

Figure 5. Formation of 8-OHdG by quartz with or without coating. Immunocytochemical appearance of 8-OHdG in A549 cells, following 4 h treatment (80 μg/cm^2) with quartz (B), or quartz treated with aluminum lactate (C) or PVNO (D), respectively. Panel A shows the control incubation.

whereas quartz coated with PVNO was found in less than 15% of the cells observed. The aluminum lactate coated particles were ingested by the cells to a larger extent in comparison to the PVNO-coated particles, however, markedly less when compared to the noncoated particles at 2 and 4 h. Interestingly, at 24 h, the particle uptake appeared to be similar for the noncoated quartz and for the aluminum-lactate-coated quartz, whereas cellular uptake of PVNO-coated particles remained minimal. Similar observations are made for the number of particles that were ingested (see Table 1).

Discussion

The proposed carcinogenicity of crystalline silica (quartz) is considered to depend on "inherent characteristics of the material" (*3*, *4*, *9*). Both in vitro and in vivo studies with quartz using coating materials such as PVNO or aluminum lactate, have demonstrated that its surface properties play an important role in its cytotoxic, inflammogenic and/or fibrogenic effect (*36*−*42*). In the present study, we demonstrate that DNA strand breakage and formation of 8-OHdG by respirable quartz in human lung epithelial cells can be inhibited following coating of its surface with aluminum lactate or PVNO. Coating also reduced the radical generation capacities of the quartz, cytotoxicity, and cellular uptake by human lung epithelial cells.

In the present study, we showed that the hydroxyl-radical formation by DQ12 was significantly, albeit incompletely, inhibited upon surface modification with aluminum lactate or PVNO. Our observations are in line with previous studies in which various ways of chemical or mechanical modification of the quartz surface were found to affect radical generation (*9*). In the past, both PVNO and aluminum lactate have been tested for their abilities to prevent or treat mineral dust-induced fibrosis, however with limited success (*40*, *44*, *56*, *57*). In the present study, however, we used both compounds to coat the surface of the quartz in order to evaluate their implications for DNA-damage. PVNO is considered to adsorb to quartz via H-bonding of its NO groups with silanol groups (*8*, *57*, *58*). This coating phenomenon has been linked to anti-fibrogenic effect, but others have also demonstrated antioxidant properties of the PVNO (*59*). The mechanism of action of aluminum lactate is less well understood, but it appears to affect the acidity and the solubility of the quartz, and might as such explain for its altered membranolytic potential (*8*, *39*, *58*). Aluminum has also been reported to hinder the formation of surface radicals and to block charges caused by grinding (*8*). Interestingly, thus, although both coatings are likely to exert different chemical interactions with the quartz surface, both chemical compounds appeared to reduce radical generation.

Our cytotoxicity data, within the A549 human lung epithelial cells, is in line with that of a variety of studies that have evaluated surface-coated quartz in relation to haemolytic activity of red cells, or, e.g., cytotoxicity in macrophages (*36*−*38*, *42*, *60*). The marked inhibition of the toxicity of the quartz following coating was also confirmed in our hands by morphological analysis of the cells (data not shown). In addition to amelioration of the cytotoxicity due to the surface coating of the quartz particles, we also demonstrated a clear inhibition of the DNA-damaging properties of the quartz. Importantly, DNA damage was determined at concentrations where no cytotoxicity occurred for any of the different particle preparations. As such, we can exclude the prevention of genotoxicity due to both surface coatings resulting from diminished cytotoxicity. The relevance of addressing cytotoxicity effects in genotoxicity testing has recently also been emphasized by an expert panel (*61*). The absence of toxicity as seen with the LDH assay and the MTT assay is also important in precluding apoptosis, which has been reported in A549 cells following prolonged treatment with quartz (*62*). However, this phenomenon is known to be highly dependent on the confluence of these cells as well as the presence or absence of serum (*63*, *64*). We determined DNA damage following 4 h treatment of the cells when near to confluency and without prior serum depletion. More importantly, we also focused on the morphological appearance of the cells, using both conventional light microscopy and electron microscopy as the gold standard. In our hands, apoptosis

was not found to occur at the 4 h time point for any of the treatments (data not shown). However, this does not exclude that apoptosis might take place at later stages, in part likely due to the observed genotoxic effects at earlier time points.

To determine the involvement of ROS in the DNA breakage by the different quartz preparations, we also determined the hydroxyl-radical specific DNA lesion 8-OHdG using immunocytochemistry. Using HLPC/ECD analysis, we previously reported that quartz and other mineral dusts may cause 8-OHdG formation (*46*, *65*). In a recent genotoxicity study with asbestos, however, immunocytochemistry analysis of 8-OHdG was used to avoid the possible artificial introduction of 8-OHdG during DNA isolation and processing for HLPC/ECD measurement as debated by numerous authors (*32*). Using the same methodology, we could demonstrate that the induction of 8-OHdG by DQ12 is inhibited by both coatings, in agreement with the comet assay data. Together with the effects of the coatings on the radical generating properties of the DQ12, and our recent observations with hydroxyl radical scavengers such as DMSO or mannitol (*29*), present data provide further support for a role of surface-mediated ROS formation and intracellular oxidant generation in the genotoxicity of quartz.

In association with the observed DNA-damaging effects, we also demonstrated that both coatings dramatically affect particle uptake by the epithelial cells. Our findings indicate that cellular uptake of quartz particles may play a crucial role in its genotoxicity, although we found a differential effect of the two coatings. The significance of phagocytosis in mineral dust-induced DNA damage has also been reported for asbestos fibers. Cytochalasin, a potent inhibitor of phagocytosis, has for instance been shown to reduce 8-OHdG formation by asbestos in HL60 cells (*34*) and to inhibit DNA strand breakage by asbestos in rabbit pleural mesothelial cells (*35*). On the other hand, coating of asbestos with vitronectin, which was also demonstrated to enhance fiber uptake, was found to further enhance DNA strand breakage (*35*, *66*). In relation to these findings, it seems likely that the complete inhibition of DNA damage in the PVNO-coated quartz might be fully explained by the minimal uptake of these particles by the epithelial cells. However, for the aluminum-lactate-coated particles, genotoxicity was also prevented, despite considerably greater uptake at 4 h compared to PVNO. This indicates that the altered surface properties of the aluminum-lactate-coated quartz, as was demonstrated by ESR, are also important within the epithelial cells. Unfortunately, however, due to the relatively abundant aluminum present on the (unmodified) DQ12 quartz, we were not able to confirm by energy-dispersive X-ray analysis, whether the aluminum remained on the particle surface of the aluminum-coated quartz particles following their cellular uptake.

In relation to our present data, others have shown that dipalmitoyl phosphatidylcholine, a major component of the lung surfactant, reduces micronucleus formation in V79 fibroblasts (*27*), and DNA damage in rat alveolar macrophages, as determined by the comet assay (*28*). These data, on one hand emphasize that lung surfactant may play an important modulating role in particle genotoxicity in vivo, and on the other hand are in agreement with our observations, i.e., that coating of the particle surface impacts substantially on the genotoxic properties of quartz particles in vitro. However, the significance of radical formation and phagocytosis was not addressed in the surfactant studies. Whether the differences in hydroxyl radical formation observed here in the different quartz preparations plays a role in the different uptake by the A549 cells is not clear, but deserves further evaluation (*67*). In addition to the observed reductions in hydroxyl radical formation following coating, other physicochemical properties may be involved, including other surface radicals, negative surface charges, acidity, or hydrophobicity of the different quartz preparations (*8*, *58*).

Obviously, in view of its implications for risk assessment, the concentration that we used in our in vitro study would extrapolate to an estimated lung burden in rats of approximately 100 mg (*68*). Nevertheless, present experiments allowed us to identify new insights on the importance of the quartz surface for its toxicity. The dissimilarities, as were observed (e.g., cytotoxicity, particle uptake) with the two different surface modifications, provide an ideal basis to further evaluate mechanisms of quartz toxicity in vitro and in vivo, e.g., as in recent and current toxicological studies by us (*42*, *69*). At the same time, however, further input is required from the chemistry research field to unravel the physicochemical properties of both surface modification compounds (*8*, *58*) that might explain for the various toxicological endpoints.

In conclusion, we demonstrate that DNA strand breakage and induction of 8-OHdG by DQ12 quartz in human lung epithelial cells can be prevented by PVNO or aluminum lactate coatings. Electron spin resonance measurements and analysis of the kinetics of particle uptake for the different quartz preparations suggest that radical generation by the quartz surface and the intracellular localization of the quartz particles both contribute to DNA damage in vitro. Our results provide important support for the proposed relationship between the radical-generating activity of the quartz surface and its genotoxicity. The data also shed important light on the "quartz paradox" highlighted by IARC, namely that quartz is not uniformly carcinogenic across all industries where there is quartz exposure. Relatively simple coating treatments, one of which (the exposure of the quartz particle surface to aluminum salts) could easily occur naturally, are shown here to totally abolish the ability of quartz to both generate hydroxyl radicals and exert crucial genotoxic effects that very likely play a role in quartz carcinogenesis.

Acknowledgment. This study was supported by the Silikosegesellschaft Nordrhein Westfalen, Germany. R.D. is funded by a fellowship from the Colt Foundation. K.D. is the Transco British Lung Foundation Fellow in Air Pollution and Respiratory Health.

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TX025558U