Reactive oxygen species and caspase activation mediate silica-induced apoptosis in alveolar macrophages

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Shen, Han-Ming, Zhuo Zhang, Qi-Feng Zhang, and Choon-Nam Ong. Reactive oxygen species and caspase activation mediate silica-induced apoptosis in alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 280: L10–L17, 2001.—Alveolar macrophages (AMs) are the principal target cells of silica and occupy a key position in the pathogenesis of silica-related diseases. Silica has been found to induce apoptosis in AMs, whereas its underlying mechanisms involving the initiation and execution of apoptosis are largely unknown. The main objective of the present study was to examine the form of cell death caused by silica and the mechanisms involved. Silica-induced apoptosis in AMs was evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling assay and cell cycle/DNA content analysis. The elevated level of reactive oxygen species (ROS), caspase-9 and caspase-3 activation, and poly(ADP-ribose) polymerase (PARP) cleavage in silica-treated AMs were also determined. The results showed that there was a temporal pattern of apoptotic events in silica-treated AMs, starting with ROS formation and followed by caspase-9 and caspase-3 activation, PARP cleavage, and DNA fragmentation. Silica-induced apoptosis was significantly attenuated by a caspase-3 inhibitor, N-acetyl-Asp-Glu-Val-Asp aldehyde, and ebselen, a potent antioxidant. These findings suggest that apoptosis is an important form of cell death caused by silica exposure in which the elevated ROS level that results from silica exposure may act as an initiator, leading to caspase activation and PARP cleavage to execute the apoptotic process.

CRISTALLINE SILICA is among the most common and potent occupational fibrogenic agents capable of inducing lung fibrosis and many other lung diseases including lung cancer (11, 33). Although the exact mechanisms involved in the development of pulmonary silicosis and silica-related carcinogenesis have not been fully elucidated, it is generally believed that 1) alveolar macrophages (AMs) are the principal target cells of silica and that they play a critical role in the fibrogenic process in silicosis (11, 26) and 2) reactive oxygen species (ROS) and oxidative damage are closely involved in silica-induced pulmonary damage (33, 39). The importance of AMs is primarily due to their unique role in mediating the interactions between inhaled particulates and various cell types such as lymphocytes and fibroblasts through the release of a wide variety of inflammatory and growth-mediating factors as well as cytokines (17).

Apoptosis is a distinct form of cell death characterized by cell shrinkage, plasma membrane blebbing, nuclear chromatin condensation, and DNA fragmentation. Different from necrosis, apoptosis is a genetically controlled active cell death process implicated as a critical physiological or pathological mechanism in development and tissue homeostasis as well as in many diseases including cancer (40). Although the details of the mechanisms of apoptosis have not been fully established, a group of cytokine proteases have been identified in both Caenorhabditis elegans (ced-3) and mammalian cells (caspases) as playing a critical role in the apoptotic process (5, 9, 37). Among the various caspases, caspase-9 and caspase-3 appear to be particularly important (22, 29). First, procaspase-9 forms an essential part of the “apoptosome,” resulting in caspase-9 activation (14, 24). The activated caspase-9 cleaves downstream caspases such as caspase-3 to orchestrate the biochemical execution of apoptosis. Second, caspase-3 is one of the major effector caspases and plays a critical role in the characteristic apoptotic changes including chromatin condensation, DNA fragmentation, and formation of apoptotic bodies (29).

Although some preliminary studies (19, 20, 23, 30) have shown that silica is able to induce apoptosis in AMs, relatively little is known about the underlying mechanisms involved. Based on the facts that silica induces ROS formation and oxidative stress in AMs and that oxidative stress is an important mediator of apoptosis, it is therefore of interest to investigate the role of ROS and oxidative stress in mediating silica-induced apoptosis in cultured rat AMs. Data from this report demonstrate that silica-induced oxidative stress initiates caspase activation and then triggers the execution of apoptosis.
Experimental Procedures

Chemicals. Standard crystalline silica was obtained from the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine (Beijing, China) and has been well characterized as being endotoxin free. More than 95% of the particulates were <5 μm in diameter. Lucigenin was purchased from Molecular Probes (Eugene, OR). RPMI 1640 medium and fetal bovine serum were from Gibco BRL (Life Technologies, Gaithersburg, MD). Ebselen was kindly provided by Rhone-Poulenc Rorer. The caspase-9 substrate N-acetyl-Leu-Glu-Asp 7-amino-4-trifuoromethyl coumarin (Ac-LEHD-AFC), 7.5% Ready Gel, and the protein quantification assay kit were from Bio-Rad Laboratories (Hercules, CA). The caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp 7-amino-4-methylcoumarin (Ac-DEVD-AMC) and caspase-3 inhibitor N-acetyl-Asp-Glu-Val-Asp aldehyde (Ac-DEVD-CHO) were from PharMingen (San Diego, CA). Anti-poly-ADP-ribose polymerase (PARP) was from BIOMOL (Plymouth Meeting, PA). Secondary anti-mouse IgG (peroxidase conjugated) and chemiluminescent substrate were purchased from Pierce (Rockford, IL). Other common chemicals and reagents were purchased from Sigma (St. Louis, MO) and were of analytic or HPLC grade.

Cell culture and treatment. Male Sprague-Dawley rats (body weight 200–220 g) were provided by the Animal Center, National University of Singapore. AMs were collected by lung lavage as previously described (42). After the lavage, the AMs were washed with PBS twice (800 g for 5 min at 37°C) and cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a 5% CO₂ incubator at 37°C. Stock ebselen (50 mM) was prepared in DMSO, and it was administered simultaneously with silica at a final concentration of 25 μM. The caspase-3 inhibitor Ac-DEVD-CHO (50 μM) was pretreated with AMs for 1 h before silica exposure.

Lucigenin-dependent chemiluminescence test. Lucigenin-dependent chemiluminescence (CL) measures the formation of superoxide anion radical (O₂⁻) in AMs. The test was carried out according to an established protocol (32) in our laboratory. The stock solution of 10 mM lucigenin was prepared in PBS and stored at −20°C in the dark. The basic reaction mixture contained 1 × 10⁶ cells and 100 μM lucigenin in 1 ml of PBS. The CL reaction was initiated by the addition of silica, and the CL level was monitored as relative light units (RLU) in a luminometer for a total period of 10 min.

2',7'-Dichlorofluorescein diacetate fluorescence test. The level of hydrogen peroxide (H₂O₂) in AMs was determined with the 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence test based on the method described earlier (31). Stock DCFH-DA (5 mM) was dissolved in ethanol and kept at −70°C in the dark. AMs were incubated in 24-well plates, each well containing 1.5 × 10⁶ AMs and 2 μM DCFH-DA in 2 ml of medium. The reaction was initiated by the addition of silica particles and incubated at 37°C for up to 4 h. The fluorescence intensity was measured with a plate reader (Tecan Spectrafluo Plus) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Determination of caspase-9 and caspase-3 activities in silica-treated AMs. Caspase-9 and caspase-3 activities were determined with their cell-permeable synthetic substrates Ac-LEHD-AFC and Ac-DEVD-AMC, respectively. After the designated treatments, AMs were collected from culture flasks and washed twice with PBS before they were lysed in a cell lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM NaH₂PO₄ (pH 7.5), 130 mM NaCl, 1% Triton X-100, and 10 mM sodium pyrophosphate]. Both substrates were reconstituted in sterile double-distilled H₂O (1 μg/μl) and added to the cell lystate (10 μg/ml). The reaction mixture was incubated at 37°C for 1 h in the dark. The fluorescence intensity of AMC or AFC liberated from the substrate was measured with spectrofluorometry (excitation at 390 nm and emission at 510 nm for AFC; excitation at 380 nm and emission at 440 nm for AMC; Perkin-Elmer LS-5B).

PARP cleavage. PARP cleavage was detected by Western blot. After treatment, the cells were collected and lysed in cell lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin. After denaturation, the samples (30 μg/well) were run on a 7.5% Ready Gel (Bio-Rad) and transferred to a nitrocellulose membrane. Immunoblotting was performed with incubation of the mouse monoclonal anti-PARP (1:5,000) primary antibody followed by incubation with anti-mouse IgG (peroxidase conjugated, 1:5,000). Signal detection was carried out with an enhanced chemiluminescence system and visualized by autoradiography.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling assay. DNA fragmentation in apoptosis was determined by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay. The experiment was conducted as reported earlier (32) with minor modifications. After treatment, AMs were collected, washed, and resuspended in PBS with 1% BSA before being fixed with 2% paraformaldehyde for 30 min at room temperature. The fixed cells were then permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 3 min on ice. The TUNEL reaction took place with addition of the reaction mixture (containing nucleotides and TdT enzyme) and was incubated for 60 min at 37°C in the dark. After one wash with PBS, the cells were finally resuspended in PBS for flow cytometry analysis (Coulter Epics Elite ESP, Miami, FL). At least 10,000 cells were collected in each group. In addition, the changes in cell size (forward scatter) and cell granularity (side scatter) of AMs were also analyzed and recorded. The signal was discriminated against silica particulates and cell debris based on the size. A negative control without the addition of TdT enzymes was always included in each experiment. The data obtained from flow cytometry were analyzed with WinMDI 2.7 software.

Measurement of cell cycle/DNA content. It is well established that DNA fragmentation during apoptosis may lead to extensive loss of DNA content and result in a distinctive sub-G₁ peak when analyzed with flow cytometry (27). The cells were first fixed and permeabilized with 70% ice-cold ethanol for >2 h followed by incubation with the freshly prepared staining buffer (0.1% Triton X-100 in PBS, 200 μg/ml of RNase A, and 20 μg/ml of propidium iodide) for 15 min at 37°C. Cell cycle was analyzed with flow cytometry with at least 10,000 cells for each sample. The histogram was abstracted, and the percentage of cells in the sub-G₁ phase was then calculated with WinMDI 2.7 software.

Statistical analysis. All data were based on at least three independent experiments; the numerical data are means ± SD and were analyzed with one-way ANOVA with Scheffe’s test. A P value < 0.05 was considered significant.
RESULTS

Silica-induced ROS formation in AMs. The results in Fig. 1A show that silica-induced $O_2^\cdot -$ formation was both time and dose dependent. A significant difference between the silica-treated and control groups was observed from the first minute onward. At the end of incubation (10 min), the RLU in AMs treated with the highest concentration of silica (100 $\mu$g/10^6 cells) was more than five times higher than that in the control cells (12,520 vs. 2,240 RLU). Meanwhile, $O_2^\cdot -$ production in the control cells was maintained at a consistent low level throughout the period. Figure 1B shows the time- and dose-dependent changes in $H_2O_2$ formation in silica-treated AMs as determined by the DCFH-DA test. Compared with that in the untreated control cells, all three doses of silica significantly enhanced the dichlorofluorescein (DCF) fluorescence intensity. For instance, at the end of the incubation (4 h), the DCF fluorescence intensity in AMs exposed to a high concentration of silica (100 $\mu$g/10^6 cells) was about two times higher than that in the control cells.

Silica-induced caspase-9 activation in AMs. The enhanced caspase-9 activity in silica-treated AMs was determined by the increase in AFC fluorescence liberated from its substrate Ac-LEHD-AFC. In the time-course study (Fig. 2A), caspase-9 activation was found to be an early event because it started to increase as early as 0.5 h after silica exposure. Caspase-9 activity reached a plateau from 2 h onward. In the dose-response study (Fig. 2B), elevated AFC fluorescence intensity was detected even with the lowest concentration of silica (25 $\mu$g/10^6 cells for 4 h) and increased in a dose-dependent manner.

Silica-induced caspase-3 activation in AMs. The time- and dose-dependent changes in caspase-3 activation in silica-treated AMs were studied, and the results are presented in Fig. 3, A and B, respectively. In the time-course study, caspase-3 activation was found to be an early event because it started to increase as early as 0.5 h after silica exposure. Caspase-3 activity peaked at 2 h and tended to decrease thereafter. In the dose-response study, a significant increase in AMC fluorescence intensity resulting from the cleavage of the caspase-3 substrate Ac-DEVD-AMC was detected even with the lowest concentration of silica (25 $\mu$g/10^6 cells for 4 h).

Fig. 1. Silica-induced reactive oxygen species (ROS) formation in rat alveolar macrophages (AMs) as determined by lucigenin-dependent chemiluminescence (CL) for the detection of $O_2^\cdot -$ (A) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence test for the detection of $H_2O_2$ (B). Data are means $\pm$ SD and were analyzed with 1-way ANOVA with Scheffe's test; $n = 4$ experiments. Significant difference ($P < 0.05$) from: *control at all time points; #control at respective time points.

Fig. 2. Silica-induced caspase-9 activation in rat AMs as measured by 7-amino-4-trifluoromethyl coumarin (AFC) fluorescence intensity liberated from its substrate N-acetyl-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC). A: time course. B: dose response. Data are means $\pm$ SD; $n = 3$ experiments. *Significant difference from control group, $P < 0.05$ by 1-way ANOVA with Scheffe's test.
Therefore, caspase-3 showed a trend generally consistent with that of caspase-9.

Silica-induced PARP cleavage in AMs. In the present study, silica-induced PARP cleavage in AMs was determined with Western blotting, and the results are presented in Fig. 4. Evident PARP cleavage (appearance of the 85-kDa band) was observed when cells were treated with silica (100 μg/10⁶ cells) for 1 h or when cells were exposed to silica at concentrations over 50 μg/10⁶ cells for 4 h.

Silica-induced apoptosis in AMs. In this study, silica-induced apoptosis was evaluated based on the following three measurements: 1) TUNEL assay, 2) cell-cycle/DNA content analysis, and 3) changes in cell size and cell granularity. Figure 5A presents the histograms of both control and silica-treated AMs in the TUNEL assay, showing the increase in TUNEL-positive cells in silica-treated AMs. The time- and dose-dependent increases in TUNEL-positive cells resulting from silica exposure are presented in Fig. 5, B and C, respectively. When treated with a high concentration of silica (100 μg/10⁶ cells), a significant increase in the number of apoptotic cells was observed from 1 h onward and >20% of the cells were apoptotic when treated for 4 h. When the silica concentration increased up to 200 μg/10⁶ cells, ~40% cells were found to be
Moreover, silica-induced apoptosis was further assessed by the determination of cell cycle/DNA content analysis. As shown in Fig. 6A, a distinctive peak of sub-G₁ cells was observed in silica-treated cells, whereas no such peak existed in the control group (inset). The percentage of sub-G₁ cells was also found to be time and dose dependent as shown in Fig. 6, B and C, respectively, similar to the patterns observed in the TUNEL assay. Last, the results shown in Fig. 7 demonstrate the reduced cell size (forward scatter) and increased cell granularity (side scatter), other characteristic changes of apoptosis, in silica-treated AMs.

Effects of Ac-DEVD-CHO on silica-induced caspase-3 activation and apoptosis in AMs. Figure 8A shows that pretreatment of the cells with Ac-DEVD-CHO (50 μM for 2 h) before silica exposure almost completely suppressed caspase-3 activation because the caspase-3 activity in that group was nearly the same as in the control cells. Moreover, Ac-DEVD-CHO also markedly inhibited silica-induced apoptosis in AMs as determined by TUNEL assay (Fig. 8B), thus indicating the involvement of caspase-3 activation in silica-induced apoptosis in AMs. In addition, silica-induced lactate dehydrogenase leakage from AMs, used as an index of cell viability, was also significantly lessened in the presence of Ac-DEVD-CHO (data not shown).

Protective effects of ebselen against silica-induced oxidative stress, caspase-3 activation, and apoptosis in AMs. The inhibitory effects of ebselen against silica-induced ROS formation as determined by the lucigenin-dependent CL and DCFH-DA fluorescence tests are presented in Fig. 9, A and B, respectively. Ebselen not only completely abolished silica-induced O₂⁻ formation in AMs but also markedly reduced the O₂⁻ level in the control cells. Ebselen was also able to significantly lower the H₂O₂ content in silica-treated AMs, although cells treated with both silica and ebselen still had a higher level of H₂O₂ than the control cells. The suppressing effect of ebselen on silica-induced caspase-3 activation was also investigated, and the results are presented in Fig. 9C. It was found that ebselen markedly reduced caspase-3 activity compared with that in the silica-only group. Figure 9D shows the results of
It is well known that ROS and oxidative damage are common and important mediators of apoptosis based on the following observations: 1) the addition or induction of ROS leads to apoptosis, 2) the depletion of cellular antioxidants promotes apoptosis, 3) apoptosis is blocked by antioxidants, and 4) many apoptosis-regulating proteins such as Bcl-2 and p53 act through the oxidant-antioxidant pathway (2, 6, 21, 25, 28). On the other hand, there is substantial evidence showing

the TUNEL assay in the presence of ebselen. Ebselen significantly reduced the percentage of TUNEL-positive cells in silica-exposed AMs, whereas ebselen alone did not cause any evident effect on apoptosis. Moreover, ebselen was also able to significantly inhibit lactate dehydrogenase leakage in silica-treated AMs (data not shown).

DISCUSSION

Although silica is known to be highly toxic to AMs, the form of cell death in silica-treated AMs has not been well studied. In a pioneering study, Sarid et al. (30) reported that silica induces apoptosis in macrophages, and subsequent studies (19, 20, 23) confirmed such a phenomenon under both in vivo and in vitro conditions as detected by morphological alterations and/or DNA gel electrophoresis. In the present study, silica-induced apoptosis in AMs was demonstrated by 1) a time- and dose-dependent increase in TUNEL-positive cells, 2) a time- and dose-dependent increase in sub-G1 cells, and 3) reduced cell size and increased cell granularity. Comparatively, the TUNEL assay with flow cytometry as used in this study is believed to be a specific and sensitive method for measuring DNA fragmentation, a hallmark of apoptosis quantitatively (1, 12). Results from this study therefore provide convincing evidence that apoptosis is an important form of cell death in silica-exposed AMs.
that ROS and oxidative stress play an important role in silica-induced pulmonary cell injury. First, silica promotes the production of various species of ROS in cell-free systems as well as in silica-exposed cells or tissues (33, 38). Results from our study also confirm that silica exposure enhances ROS production in cultured rat AMs (Fig. 1). Second, silica is able to induce oxidative damage such as lipid peroxidation and oxidative DNA damage both in vivo and in vitro (10, 34). Third, many antioxidants are capable of protecting against silica-induced cytotoxicity (18, 35). Therefore, it will be of interest to explore the involvement of ROS and oxidative stress in silica-induced apoptosis. In this study, it was noted that a significant increase in ROS formation preceded other apoptotic events such as caspase-3 activation and DNA fragmentation. Ebselen, a potent antioxidant and ROS scavenger (36, 41), was able to reduce the ROS level, inhibit caspase-3 activation, and eventually protect against silica-induced apoptosis (Fig. 9). It appears that ROS and oxidative stress may act as initiating factors in silica-induced apoptosis in cultured rat AMs.

At present, the exact signaling pathway of apoptosis triggered by ROS and oxidative stress remains to be elucidated. An important step in apoptosis is the activation of a series of proteases called caspases. Among 14 caspases identified so far, caspase-3 has been found to be one of the most important effectors in executing apoptosis (29). In the present study, the critical role of caspase-3 activation in silica-induced apoptosis was demonstrated by 1) a time- and dose-dependent increase in caspase-3 activity in silica-treated AMs, 2) evident PARP cleavage, and, more importantly, 3) the protective effect of Ac-DEVD-CHO, a specific caspase-3 inhibitor, against silica-induced apoptosis. Results obtained from this study are generally consistent with those reported previously that caspase-3 or cysteine protease protein-32 was involved in silica-induced apoptosis in human macrophages in vitro (20). Furthermore, the present study also evaluated the activation of caspase-9, an upstream caspase that is activated by the formation of apoptosome with cytochrome c, apoptotic protease-activating factor-1, and dATP (14, 24). Although no direct evidence is currently available, it is believed that the elevated level of ROS generated from silica exposure may initiate an apoptotic pathway through mitochondria, causing cytochrome c release and caspase-9 activation, which subsequently lead to caspase-3 activation, PARP cleavage, and DNA fragmentation.

On the other hand, based on the fact that both the caspase-3 inhibitor Ac-DEVD-CHO and the antioxidant ebselen failed to completely block the occurrence of apoptosis, it is believed that there are some other intermediate factors regulating the signaling pathway from ROS formation to caspase activation and DNA fragmentation. At present, it has been extensively studied that silica is able to cause nuclear factor (NF)-κB activation, most probably through the action of ROS (8). NF-κB is a very important nuclear transcription factor that is closely associated with the signaling pathway of apoptosis (4, 7). Moreover, ROS are also involved in the release of cytokines including tumor necrosis factor-α, interleukin-1α, and interleukin-1β from silica-treated AMs (13, 30), which, in turn, are important factors in initiating or regulating apoptosis. The exact role of NF-κB or other NFs in silica-induced apoptosis remains to be further investigated.

At present, the biological relevance of silica-induced apoptosis to the development of pulmonary diseases such as silicosis and carcinogenesis has not been well defined. Based on the observation that crystalline silica-induced apoptosis in AMs, whereas no similar effects were found with amorphous silica or titanium dioxide, it has been proposed that the fibrotic potential of a particulate depends on its ability to cause apoptosis (19). Such a proposal was obviously supported by studies on other fibrotic agents such as bleomycin and asbestos that are able to induce apoptosis in AMs as well (15, 16). It is well known that an increase in recruitment and activation of AMs plays an important role in the pathogenesis of lung disease resulting from silica exposure (11, 26). Apoptosis has been identified to be a critical pathway for the elimination of damaged or injured cells and for the maintenance of tissue homeostasis. By studying silica-induced apoptosis in AMs and granulomatous cells under an in vivo condition, Leigh et al. (23) believed that silica-induced apoptosis has a regulatory role in the process of inflammation in silica-exposed lung tissue by the following two mechanisms: 1) attracting more AMs into the alveolar space to engulf apoptotic cells and 2) maintaining a relatively stable level of neutrophils in the inflammatory sites. On the other hand, silica-induced apoptosis may also be implied in the carcinogenic process, and the exact mechanisms involved remain to be further elucidated.

Ebselen is a synthetic heterocyclic selenoorganic compound with potent antioxidant activity. Basically, it mimics the reactions of glutathione peroxidase, with thiols as electronic donors (36). It is well known that ebselen is able to protect against oxidative damage such as lipid peroxidation both in vivo and in vitro as well as to scavenge free radicals including superoxide radicals, hydroxyl radicals, peroxyl radicals, and peroxynitrite (3, 36, 41). In the present study, ebselen was able to protect against silica-induced apoptosis in AMs, most probably through its potent antioxidant activity, by scavenging ROS formed in silica-treated AMs. Therefore, results from this study suggest that ebselen might have the potential as a preventive agent against silica-induced pulmonary injury.

In summary, results from this study suggest that apoptosis is an important form of cell death caused by silica exposure. The elevated ROS level resulting from silica exposure may act as an initiator, leading to caspase-9 and caspase-3 activation and PARP cleavage to execute the apoptotic process. Knowledge of such a signaling pathway may help to obtain a better understanding of the underlying mechanism in the development of silica-induced pathological changes.
REFERENCES


