Surfactant protein A prevents silica-mediated toxicity to rat alveolar macrophages

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Silicosis is a major occupational lung disease caused by the inhalation of crystalline silica [silicon dioxide (SiO2)], a cytotoxic and fibrogenic mineral dust (3, 46). The reasons for the development of silicosis and fibrosis are not well understood. However, the interaction between crystalline silica and the alveolar macrophage (AM) is generally considered to be an early and critical step that initiates a cascade of events resulting in histopathological evidence of injury and fibrosis (1, 6, 10, 27). In a recent in vivo study (52) examining the response of AMs and interstitial macrophages to the airway delivery of silica, AMs were by far the predominant type of macrophage engulfing silica particles. Although AMs attempt to protect the lung by ingesting the silica particulates from the alveolar spaces, silica continues to cause injury and promote release of toxic radicals, inflammatory mediators, and chemotaxins that contribute to the pathogenesis of disease (10, 17).

Silica, primarily α-quartz, is cytotoxic to AMs in vitro (9, 22, 33, 39, 47). Direct toxicity to cells such as AMs is one proposed mechanism by which silica is thought to initiate an inflammatory response in silicosis (10). The mechanism by which silica exerts its toxic effect on AMs is unclear, although recent studies (5, 11, 14, 20, 24, 38, 42, 43) indicated that cellular injury may be mediated in part by oxygen- and/or nitrogen-derived free radicals.

Evidence from both animal models (4, 21, 40, 41) and humans (9) suggests that silica exposure results in acute cellular injury in vivo (4, 9, 21, 40, 41). However, silica-induced injury in vivo appears to be markedly diminished compared with injury in vitro (7, 12, 18). The reason for this relative protection from silica-induced injury in vivo is unknown. It is well recognized that after acute silica exposure, an excessive amount of surfactant accumulates in the lungs (8, 19, 23, 25). Similarly, there is a marked elevation in surfactant apoprotein (SP) A, SP-B, and SP-D (13, 29, 31, 36, 45). Previous studies have shown that surfactant phospholipids diminish silica-mediated cytotoxicity in vitro (2, 22, 39, 47) and in vivo (2). However, the possible role of SPs in this initial silica-AM interaction is unstudied.

SP-A is the major protein component of alveolar surfactant. It is a C-type lectin possessing a carbohydrate recognition domain and is structurally homologous to C1q (26). SP-A has been shown to specifically attach to AMs (32, 37), and there is substantial evidence to indicate that SP-A plays an important role in host defense, especially in the response of AMs to microorganisms (28, 44, 51). Recently, a study (35) indicated that SP-A as well as SP-D is upregulated during acute inflammation in the lung, suggesting that SPs may act as acute-phase reactants similar to mannoside binding protein in the serum.

In this study, in vitro cytotoxicity of silica to rat AMs was quantified with a 51Cr release cytotoxicity assay (34). Silica-mediated toxicity to AMs was dependent on the duration of incubation and the concentration and size of the silica particles. Silica directly injured rat AMs as evidenced by a cytotoxic index of 32.9 ± 2.5, whereas the addition of rat SP-A (5 µg/ml) significantly reduced the cytotoxic index to 16.6 ± 1.2 (P < 0.001). This effect was reversed when SP-A was incubated with either polyclonal rabbit anti-rat SP-A antibody or D-mannose. These data indicate that SP-A mitigates the effect of silica on AM viability, and this effect may involve the carbohydrate recognition domain of SP-A. The elevation of SP-A in acute silicosis may serve as a normal host response to prevent lung cell injury after exposure to silica.
of the silica. SP-A reduced the direct cytotoxic effect of silica on AMs, and this protective effect was reversed by the addition of α-mannose, a sugar that has high affinity for the carbohydrate recognition domain, and by anti-SP-A polyclonal antibody. These data support the hypothesis that the elevation in SP-A after acute exposure to silica may represent an appropriate host response that occurs in vivo to limit the injurious effect of silica on specific lung cells.

METHODS

Isolation and culture of AMs. Pathogen-free female Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were housed in open cages and killed with an intraperitoneal injection of Beuthanasia-D solution (Schering-Plough Animal Health, Kenilworth, NJ). The trachea was cannulated after a midline neck incision was made, and the lungs were lavaged six to seven times with 8- to 10-ml aliquots of Hank’s balanced salt solution without Ca2+ or Mg2+ (GIBCO BRL, Life Technologies, Grand Island, NY) plus 0.6 mM EDTA, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Approximately 50 ml of fluid were obtained from each rat. Lavage fluid was centrifuged (1,200 g for 5 min), and the supernatant was discarded. To preferentially remove any red blood cells, the cells were suspended in lysing solution containing 0.01 M KHCO3 and 0.15 M NH4Cl and repelleted. The cells were resuspended in 0.9% NaCl, and cell number was determined with hemocytometer counts. A cell differential was performed by examination of cytopreparation smears (Cytospin II, Shandon Southern Instruments, Sewickley, PA) that demonstrated that >95% of the cells were AMs. AMs were suspended in Dulbecco’s modified Eagle’s medium (DMEM; BioWhittaker Bioproducts, Walkersville, MD) supplemented with glutamine (0.6 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (4 µg/ml), amphotericin B (0.5 µg/ml), and 20 mM HEPES (pH 7.4). The AMs were then plated on 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) at a density of 2.5 × 103 cells/well in 0.2 ml of DMEM. The cells were allowed to adhere for a minimum of 4 h at 37°C in a 5% CO2 atmosphere. Before use, monolayer AMs were gently washed with DMEM to remove any nonadherent cells.

Silica. Crystalline silica was obtained from US Silica (Berkeley Springs, WV). The purity and α- quartz structure of the crystals were confirmed by X-ray powder diffraction (Scintag XDS-2000 powder diffractometer, Scintag, Cupertino, CA). Silica was fractionated by size with repetitive centrifugation in distilled water at graded speeds. Silica crystal diameter was determined by averaging the length of the long and short axes as previously described (49). Averages were determined from 100 crystal measurements/fraction. Size fractions were confirmed by fluorescence-activated cell sorting analysis (FACStar Plus flow cytometer and FACScan analyzer, Becton Dickinson, Mountain View, CA). Crystal sizes were 18 ± 6 µm for fraction 1, 8 ± 3 µm for fraction 2, 4 ± 1.5 µm for fraction 3, and 2 ± 1 µm for fraction 4. Each fraction was air-dried and then heated to 250°C for 2 h to destroy endotoxin. The crystals were suspended in Hanks’ balanced salt solution or DMEM for all experiments. Aluminum oxide (Al2O3) in the form of corundum (Aldrich, Milwaukee, WI) was used as a nontoxic inorganic crystal control with a mean crystal size of 2.4 µm (48).

SP-A and anti-SP-A antibody preparation. Rat SP-A and anti-SP-A polyclonal antibody production (rabbit anti-rat) were prepared as previously described (50, 51). Levels of endotoxin in SP-A preparations were determined by quantitat-
induced cytotoxicity to cultured AMs in a concentration- and time-dependent manner (Fig. 1). Silica at concentrations of 16, 31, 62, 125, and 250 µg/ml was incubated with 51Cr-labeled AMs for 2, 4, 6, 8, 10, and 12 h. The results show that each concentration of silica plateaued between 4 and 6 h and that the CI is directly proportional to the concentration of the silica.

When the silica crystals were sorted by size (<2, 3–6, 5–11, and 12–24 µm), smaller crystals were more toxic to AMs than larger crystals with quantities of equivalent mass. For example, 12- to 24-µm silica crystals resulted in a CI of −0.103 ± 0.803, whereas those <2 µm resulted in a CI of 61.3 ± 1.853 (P < 0.05; Fig. 2A). As expected, each progressively smaller size fraction of silica was associated with a greater surface area (Fig. 2B). These results were used to determine the relationship between surface area and CI (Fig. 2C). We next determined the influence of particle size with an equivalent surface area for each fraction on AM cytotoxicity (1.6 cm²). For the smaller silica particles (3–6 and <2 µm), the CIs were 20.8 ± 0.9 and 32.7 ± 0.5, respectively (P < 0.05; Fig. 3). For the larger particle sizes of 5–11 and 12–24 µm, there was no significant cytotoxicity, with CIs of 1.9 ± 4.0 and 1.8 ± 2.5, respectively (P > 0.05). Thus silica toxicity to AMs was concentration and time dependent. Furthermore, silica toxicity was dependent on crystal size even when the surface area was held constant, with smaller silica crystals being more toxic than larger ones. Although silica induced cytotoxicity to cultured rat AMs, Al2O3, a nonfibrogenic inorganic dust, did not.

Effect of SP-A on silica-mediated injury to AMs. SP-A significantly diminished 51Cr release from silica-exposed AMs from 32.9 ± 2.5 to 16.6 ± 1.2% (P < 0.001; Fig. 4). A protein control also slightly decreased injury from 32.9 ± 2.5 to 26.5 ± 1.7% (P < 0.05), but SP-A was much more effective than the protein control in reducing 51Cr release (P < 0.001). SP-A added to AMs in the absence of silica had no effect on baseline 51Cr release by control AM monolayers (data not shown). When D-mannose (50 mM) was preincubated with SP-A for 20 min before the addition of silica and AMs, the protective effect of SP-A (CI of 16.6 ± 1.2) was lost, with a return in the CI to 27.9 ± 2.6 (P < 0.01; Fig. 4). Preincubation of SP-A with polyclonal anti-SP-A antibody (2.5 µg/ml) had an effect similar to D-mannose in reversing the
protective effect of SP-A on silica-mediated cytotoxicity. Anti-SP-A antibody reversed the SP-A inhibitory effect, from a CI of 16.6 ± 1.2 to 29.9 ± 3.0 (P < 0.01), a value essentially equivalent to that of AMs incubated with silica alone (32.9 ± 1.5; P = 0.339). Anti-SP-A antibody, nonspecific IgG antibody, and D-mannose alone showed no significant effect on 51Cr release by AMs (data not shown).

Confirmation of these findings was provided by morphological assessment of the cultured AMs in the presence and absence of silica with and without SP-A. With phase microscopy, silica particles were easily visible (Fig. 5). AMs in the presence of silica revealed fewer cells to be present compared with those without silica, and the AMs demonstrated typical findings of cell injury with rounding and increased light refraction, suggesting imminent lifting of the cells from the plate. SP-A significantly attenuated loss of AMs and reduced injured appearance of AMs remaining on the plate.
DISCUSSION

The effect of crystalline silica on AMs has been extensively studied (1, 5, 6, 10, 14, 17, 20, 22, 24, 27, 33, 38, 42, 43, 47). Our study indicates that silica toxicity to AMs can be quantified by use of a 51Cr assay and that the injury to AMs is dependent on the concentration and size of silica particles as well as on the time of incubation. SP-A significantly reduced the injury to AMs by silica. Interestingly, the data suggest that the carbohydrate recognition domain of SP-A is responsible for this protection. Taken as a whole, the elevation in SP-A in response to acute silica exposure may represent an appropriate host response to mitigate the injurious effects of harmful silica particles that reach the alveolar spaces.

Crystal size has been implicated as an important factor in the biological effect of silica. However, the relationship of crystal size to silica-mediated injury to AMs is unclear. Silica crystals that are 1 µm in diameter have been shown to have the greatest membrane-lytic effect on red blood cells (49). Conversely, crystals ≥ 5 µm in diameter have been shown to be the most fibrogenic (49). In our study, crystals ≤ 6 µm were found to have the greatest ability to injure cultured AMs. This is in agreement with a previous study by Allison et al. (1) that indicated that silica crystals of respirable size (0.5–5 µm) are highly toxic to cultured peritoneal macrophages. Smaller crystal size fractions (<2 and 3–6 µm) were more cytotoxic to AMs even when an equivalent surface area was used (Fig. 3). This occurred even though the mass of the silica particles was less in the smaller size fractions.

Several investigators have suggested that surfactant phospholipids can diminish the cytotoxicity of α-quartz to AMs in vitro (22, 39, 47) and in vivo (2). This has been proposed as one mechanism by which direct AM injury by silica in vivo can be attenuated (18). A recent study (30) has indicated that Survanta, a mixture of both bovine phospholipids and SPs, delivered intratracheally provides in vivo protection to the lung from silica-induced release of inflammatory mediators. It is unclear, however, to what extent SPs may play a role in diminishing the cytotoxic effects of silica.

It is well known that acute silica exposure in animals greatly increases SP-A levels because airway delivery of silica is the standard method used for the isolation and purification of SP-A from animals (13, 29, 31, 36, 45). However, the direct effect of SP-A, the major protein component of surfactant, on silica-mediated injury to AMs has not been previously studied. In our study, SP-A clearly protected AMs from the injurious effects of silica as evidenced by a standard cytotoxicity marker and phase microscopy. The protection was not complete, however, suggesting that other factors such as the presence of phospholipids, as discussed above, or perhaps other protein components such as SP-D are also important in providing protection in vivo. The ability of an SP-A antibody to reverse this protection provides evidence that a specific site on the SP-A molecule may be responsible for this protective effect.

D-Mannose is a sugar that exhibits high affinity for the carbohydrate recognition domain of SP-A (26). The finding that D-mannose reverses the effect of SP-A on silica-mediated AM injury suggests that the carbohydrate recognition domain of SP-A may play a role in this protective effect from silica.

Although our study suggests that SP-A can be protective in acute silica exposure in vitro, it is less clear whether protection of AMs in vivo will necessarily be in the best interest of the host. SP-A might serve to prolong the viability of AMs activated by silica, thereby increasing the number of AMs actively releasing the proinflammatory mediators and chemotaxins that are thought to be important in the pathogenesis of disease (17). If true, SP-A might promote the disease process even as it affords some protection to the AMs. On the other hand, SP-A may be protective to cells other than simply to AMs. Such cells could include alveolar epithelial cells; in this case, a protective role for SP-A is thought to be important in the pathogenesis of disease. In summary, this study demonstrated that silica crystals are directly cytotoxic to rat AMs and that the degree of cytotoxicity is dependent on the size and concentration of crystalline silica as well as on the duration of exposure. This study indicated that SP-A reduced silica-mediated AM injury in vitro and that this effect likely involves the carbohydrate recognition domain of the SP-A molecule. These data are consistent with the hypothesis that elevated SP-A levels may serve as an appropriate host response to prevent further cell injury after acute exposure to silica. Whether SP-A elevation in vivo in response to acute silica exposure is actually beneficial to the host animal is unknown and requires further study.

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