Published in final edited form as:

J Toxicol Environ Health A. 2010 January ; 73(10): 669–683. doi:10.1080/15287390903578521.

Granuloma Formation Induced by Low-Dose Chronic Silica inhalation is Associated with an Anti-Apoptotic response in Lewis Rats*

Raymond J. Langley, Neerad C. Mishra, Juan Carlos Peña-Philippides, Julie A. Hutt, and Mohan L. Sopori[†]

Immunology Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr., SE, Albuquerque, NM 87108

Abstract

Chronic human silicosis results primarily from continued occupational exposure to silica and exhibits a long asymptomatic latency. Similarly, continued exposure of Lewis rats to low doses of silica is known to cause delayed granuloma formation with limited lung inflammation and injury. On the other hand, intratracheal exposure to large doses of silica induces acute silicosis characterized by granuloma-like formations in the lung associated with apoptosis, severe alveolitis, and alveolar lipoproteinosis. To ascertain similarities/differences between acute and chronic silicosis, in this communication, we compared cellular and molecular changes in established rat models of acute and chronic silicosis. In Lewis rats, acute silicosis was induced by intratracheal instillation of 35 mg silica, and chronic silicosis through inhalation of aerosolized silica (6.2 mg/m³, 5 days/week for 6 weeks). Animals exposed to acute high-dose silica were sacrificed at 14 days after silica instillation while chronically silica-treated animals were sacrificed between 4 days and 28 wk after silica exposure. The lung granulomas formation in acute silicosis was associated with strong inflammation, presence of TUNEL-positive cells and increases in caspase-3 activity and other molecular markers of apoptosis. On the other hand, lungs from chronically silica-exposed animals exhibited limited inflammation and increased expression of anti-apoptotic markers, including dramatic increases in Bcl-2 and procaspase-3, and lower caspase-3 activity. Moreover, chronic silicotic lungs were TUNEL-negative and overexpressed Bcl-3 and NF-κB-p50 but not NF-κB-p65 subunits. These results suggest that, unlike acute silicosis, chronic exposures to occupationally relevant doses of silica causes significantly lower lung inflammation and elevated expression of anti-apoptotic rather than proapoptotic markers in the lung that might result from interaction between NF-κB-p50 and Bcl-3.

Keywords

silicosis; apoptosis; lung inflammation; granuloma

Epidemiological studies indicate that chronic silicosis is generally associated with occupational exposure to crystalline silica and is mostly prevalent in stone dressers, and quarry and foundry workers (Vallyathan et al., 1998; Chong et al., 2006; Hnizdo and Vallyathan, 2003). Depending on the dose, silica may produce acute (accelerated silicosis) or various forms of chronic silicosis (Borges et al., 2002; Castranova et al., 2002; Chong et al., 2006; Hnizdo and Vallyathan,

*These studies were supported in part by grants from NIH (R01 DA017003, R01 DA04208-15, and R01DA04208) and from the U.S. Army (W81XWH-04-C-0071).

[†]Address correspondence and reprint requests to: Mohan Sopori, Ph.D., Immunology Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr., SE, Albuquerque, NM 87108, Tel.: (505) 348-9440; Fax: (505) 348-4986; msopori@lrri.org.

2003; Langley et al., 2004; Shi et al., 1998; McLaughlin et al., 1997). Although in animals, both high-dose acute and low-dose chronic silica exposures induce granulomatous changes in the lung, the granuloma-like structures formed in acute silicosis are a loose aggregate of activated foamy histiocytes and lymphocytes. On the other hand, the granulomas in chronic silicosis in both humans (Hnizdo and Vallyathan, 2003) and animals (Langley et al., 2004) are discrete and well organized nodular structures, consisting of epithelioid macrophages and multinucleated giant cells. Chronic silicosis is the most prevalent form of silicosis and exhibits a long latency (Shi et al., 1998; Vallyathan et al., 1998; Chong et al., 2006) and, generally, the patients remain asymptomatic for decades. The disease is eventually diagnosed by the history of occupational silica exposure and the presence of fine nodular opacities (≤ 1 cm diameter) in the lung by chest X-ray or CT scan (Hughes et al., 1998). Chronic silicosis is a progressive disease that is resistant to all known anti-inflammatory treatments, including corticosteroid therapy (Chong et al., 2006).

While the molecular events that underlie silicosis are largely unknown, human acute silicosis usually results from accidental exposures to high doses of silica. Acute silicosis is characterized by severe alveolitis, alveolar lipoproteinosis, and a progressive clinical course that often results in death (Chong et al., 2006). Similarly, experimental acute silicosis in animals is induced with intratracheal instillation of large doses of silica, and associated with apoptosis, severe lung inflammation, alveolar lipoproteinosis, increased arginase expression, and tissue destruction (Borges et al., 2002; Zhang et al., 2002; Poljakovic et al., 2007). These findings led to the general belief that apoptosis may be the critical event in the development of silicosis; however, evidence from human patients with the most prevalent form of silicosis (i.e., chronic silicosis) does not suggest a significant role of apoptosis in the development of lung granulomas (Tomokuni et al., 1997). In the rat model of chronic silicosis, where animals were exposed to occupationally relevant doses of silica (Hnizdo and Sluis-Cremer, 1993), it was shown that granuloma formation in the lung occurs months after chronic low-dose silica exposure with limited degree of lung inflammation (Langley et al., 2004). Similar to human silicosis (Williams and Williams, 1983), in this model, the lung granulomas contain multinucleated giant cells composed of silica containing epithelioid macrophages. The aim of this study was to use established rat models of acute and chronic silicosis and present evidence that, unlike the granulomas formed in acute silicosis, chronic silicotic granulomas exhibit cellular and molecular markers that inhibit apoptosis.

Materials and Methods

Animals

Six- to 8-wk-old, pathogen-free, male Lewis rats, weighing 160–180 g, were purchased from Charles River Laboratories (Raleigh, NC). Prior to silica exposure, the animals were conditioned for 2 weeks to the inhalation conditions in H2000 whole-body exposure chambers (Lab Products, Inc., Maywood, NJ). After exposure animals were transferred to class-100 air quality rooms in shoebox cages with hardwood chip bedding. Food and water were provided ad libitum throughout the experimental period, and animals were periodically monitored for common rat infections. Animal care was provided in accordance with the procedures outlined in the “Guide for the Care and Use of Laboratory Animals.

Animal Exposures

To induce chronic silicotic granulomas, rats were chronically exposed to low doses of silica inhalation as described previously (Langley et al., 2004). Briefly, exposure chambers were maintained with an airflow rate of approximately 15 cfm and temperature range of 22–26°C. Rats received 6.2 mg/m³ aerosolized silica (Min-U-Sil 5; U.S. Silica, Mill Creek, OK) with an average particle size of 1.75 ± 0.05 μ m (mass median aerodynamic diameter) 6 hr/day, 5 days/

week (Monday–Friday) for 6 weeks. Control animals were exposed to filtered air under similar inhalation conditions. Although the aerosolized silica concentration during the exposure was considerably higher than the Permissible Exposure Limit of OSHA (0.05 mg/m^3), lung silica burden of these animals was even lower than the South African gold miners with silicosis (Hnizdo and Sluis-Cremer, 1993) and, therefore, occupationally relevant. The animals were sacrificed at 4 days, and at week 7, 14 or 28 post silica exposure. Our previous studies indicated that other than increased number of “foamy” macrophages, there were no other signs of significant lung inflammation between day 4 and week 28 post silica exposure (Langley et al., 2004). Macrophage aggregation (early indication of granuloma formation) was first noted at 7 weeks post silica exposure, but frank granuloma development did not occur until week 17 after silica exposure and were well established by 27 weeks after silica treatment (Langley et al., 2004). To generate the acute silicotic granulomas, we followed the established method of silica exposure was followed (Barbarin et al., 2005; Barbaro et al., 2002; Borges et al., 2001). Briefly, rats received single intratracheal instillation of 35 mg silica in 750 μl of saline or saline alone (control). These animals were sacrificed 14 days post silica exposure.

Bronchoalveolar Lavage Collection

Bronchoalveolar lavage (BAL) and BAL cells were collected as described (Langley et al., 2004). Briefly, rats were anesthetized with isoflurane-oxygen and the left lung lobe was tied off at the bronchi with clips, while the right lobe was lavaged twice with 3-ml aliquots of sterile saline solution through a tracheal cannula. The lavage was centrifuged for 5 min at $1,500 \times g$ and BAL fluid and cell pellets (BAL cells) were collected.

Preparation of Lung Tissue Sections

For immunohistochemistry (IHC), slides of lung sections were made as described (Langley et al., 2004). Briefly, the left lung was inflated for 2 hr at a constant hydrostatic pressure of 25 cm with 10% neutral buffered formalin and immersed in the same solution for 48 hr. The fixed lungs were trimmed in the dorsoventral transverse direction from cranial to caudal, embedded in paraffin, and cut into 5- μm sections. The sections were used for IHC staining and TUNEL assay.

TUNEL Assay

TUNEL staining and setting up of positive and negative controls was performed on paraffin-embedded lung sections by the TACS 2 TdT-DAB In Situ Apoptosis Detection Kit (Trevigen; Gaithersburg, MD) as per manufacturer's instructions. TUNEL positivity was quantitated by counting the number of apoptotic bodies within 3 microscopic fields of granulomatous tissue from each animal. A total of 5 animals per group were analyzed. Because the chronically silica-exposed rats do not develop frank granulomas until after 17 weeks post silica exposure (Langley et al., 2004), TUNEL assay was performed on lungs from day 4, and week 7, 14 or 28, but frank granulomas were seen only in the 28 week silicotic lungs.

Caspase-3 Immunoassay

Caspase 3 activity of the lung lysates was determined by the Quantikine ELISA immunoassay kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. Briefly, lung extracts from control, chronically silica-treated for indicated times, and 14-day acutely silica-treated animals were incubated with 1 μM staurosporine followed by incubation with 10 μM Biotin-ZVKD-fmk for 1 h. The samples were adjusted to a protein concentration of 25 $\mu\text{g/ml}$, diluted 1:10 and 1:20, and analyzed for caspase-3 activity on an ELISA reader at 450 nm and calculated as ng/ml of the lung extract.

Immunohistochemistry

Lung sections were deparaffinized, and antigen retrieval was performed using the pressure cooker method (i.e., slides were treated with 2% citric acid, pH 6.0; for 15 min at 90°C in a sealed flask). Slides were then soaked in peroxidase-blocking solution (0.5% H₂O₂) for 15 min, and incubated at room temperature for 90 min in TNK buffer (100 mM Tris, 550 mM NaCl, 10 mM KCl, 1% normal goat serum, 2% bovine serum albumin, and 0.1% Triton X-100, pH 7.6). The slides were stained overnight in various dilutions of primary antibodies in TNK buffer, including anti-Bcl-2 (BD Pharmingen; San Jose, CA), anti-NF-κB p65 (Abcam Inc; Cambridge, MA), anti-NF-κB p50, and anti-Bcl-3 (Santa Cruz Biotechnology; Santa Cruz, CA). The slides were washed with phosphate buffered saline and incubated with a 1:200 dilution of peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) for 1 hr at room temperature. Normal rabbit IgG in place of the primary antibody or only the secondary antibody were used as controls for non-specific binding. The slides were treated with the streptavidin-conjugated peroxidase detection kit (Vector Laboratory; Burlingame, CA) as per manufacturer's instructions and counterstained with hematoxylin (1:10 dilution).

Gel Electrophoresis and Immunoblotting

Western blot analysis was performed as described previously (Razani-Boroujerdi and Sopori, 2007). BAL cells were washed with PBS and lysed in ice-cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5 % sodium deoxycholate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 µg/ml each of leupeptin and aprotinin), incubated on ice for 15 min, and centrifuged. Protein concentration of the supernatants was determined by the BCA Protein Assay kit (Bio-Rad; Hercules, CA). Proteins were resolved on 12% SDS Criterion Gels (Bio-Rad) and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by incubating the membranes in the blocking buffer (5 % nonfat dry milk in 10 mM Tris and 150 mM NaCl, 0.1% Tween 20, pH 7.2) for 1 hr at room temperature. Membranes were then incubated overnight at 4°C with a 1:1000 dilution of rabbit Bcl-2, Bax, NF-κB p50/p105, NF-κB p65, GAPDH antibodies, goat anti-procaspase-3, or mouse anti-ubiquitin antibody (Santa Cruz Biotechnology). The membranes were extensively washed with TBST (Tris-buffered saline, pH 8, and containing 0.05% Tween 20). The blots were incubated with peroxidase-linked anti-rabbit, anti-goat or anti-mouse IgG (Santa Cruz Biotechnology) in TBST for 1 hr at room temperature and washed extensively with TBST. Blots were developed by chemiluminescence reagents (Amersham, Arlington Heights, IL), and the bands were quantified with a Bio-Rad GS-800 calibrated densitometer and Quantity One software.

Statistical Analysis

The results were analyzed for statistical significance using a student's *t* test by GraphPad Prism Software 3.0 (GraphPad Inc., San Diego, CA), and *p* values of ≤ 0.05 were considered significant. Error bars in the figures represent the standard error of mean.

Results

Granulomas from Acute but not Chronic Silica-exposed Animals Exhibit Apoptosis

Acute silicosis is associated with apoptosis and lung injury (Borges et al., 2002). To ascertain whether chronic silicosis is also associated with an apoptotic response, lung tissues from chronically silica-exposed animals were scored for the presence of apoptotic cells by TUNEL assay at day 4, or week 7, 14, and 28 post silica exposure. Lungs from acutely silica exposed animals at day 14 post silica exposure were used as the positive control. Figure 1 compares the TUNEL reactivity of lungs from sham control animals with those from chronically and acutely silica-exposed animals. It is apparent that while both acute and 28-week chronic silica

exposures induce lung granulomas (Figure 1A), only the acute silicotic granulomas contain numerous TUNEL-positive cells (Figure 1A, right panel). On the other hand, lung tissues from week 28 post chronic silica exposures did not exhibit any TUNEL-positive cells (Figure 1A, middle panel). Similarly, lung tissues collected from chronically silica-exposed animals at day 4, or week 7 and 14 after silica exposure also lacked TUNEL-positive cells (data not shown). This was the case with all the randomly examined tissues sections from the lung. As mentioned in the Materials and Methods, chronically silica-exposed animals show limited inflammation (leukocytic infiltration) and tissue injury (BAL LDH activity and total protein) between day 4 and week 10 after silica exposure (Langley et al., 2004). These results suggest that under these conditions of chronic silica exposure, neither the granulomas nor the lung tissues exhibit significant apoptosis or cellular injury up to 28 week after silica exposure. Thus, unlike acute silicosis, development of chronic silicotic granulomas does not require apoptotic cell death or a strong inflammatory response.

Chronic Low-dose Silica Inhalation Induces Molecular Imprints of an Anti-apoptotic Response

Because the lungs from chronically silica-exposed animals did not show significant TUNEL staining and tissue injury, studies were undertaken to determine whether chronic silica inhalation affected the molecular markers of apoptosis in BAL cells. Caspase-3, the effector caspase in apoptosis, is activated by proteolytic cleavage of its zymogen form procaspase-3 (Boatright and Salvesen, 2003). Figures 2A and 2C show that BAL cells from control animals possess low levels of procaspase-3, but the cells from chronically silica-exposed animals at week 14 after silica exposure have large quantities of this inactive form of caspase-3. On the other hand, BAL cells from acutely silica-treated animals have procaspase-3 levels that are even lower than controls (Figure 2B and 2D). Indeed, to visualize the procaspase-3 band in samples from acute silicosis (Figure 2B), the gels were loaded with quantitatively higher concentrations of the cell lysate proteins than the gels in Figure 2A, indicating that the lung procaspase-3 levels are quantitatively lower in acute silicosis, suggesting that the enzyme might exist mainly as the active caspase 3 in acute silicosis. To examine this possibility, caspase-3 activity was determined in lung lysates from control, acute silicosis (2 weeks after silica instillation), and chronic silicosis treatments at various times after silica inhalation. Results presented in Figure 3 show that compared to control lungs, chronic exposure with low doses of silica did not produce significant activation of caspase-3; on the other hand, caspase-3 activity was significantly elevated in acutely silica-exposed lungs. Increased pro-caspase concentrations were also seen in BAL cells from animals sacrificed at week 28 after chronic silica exposure (not shown). Thus, the results suggest that lungs from chronically silica-exposed animals failed to convert pro-caspase-3 into active caspase-3, leading to the accumulation of procaspase-3 and decreased effector caspase-3 activity.

The Bcl-2 family of proteins regulate cellular apoptosis, and two family members, Bcl-2 and Bax, act as important anti- and pro-apoptotic factors, respectively (Burlacu, 2003). Time course data in Figure 4A and 4B show that chronic silica exposures produced a significant increase in Bcl-2 at ≥ 7 weeks. The rise in Bcl-2 became even more robust at 14 and 28 weeks after silica exposure. Thus, chronic inhalation of silica increases the expression of anti-apoptotic Bcl-2.

The anti-apoptotic effect of Bcl-2 is mitigated by pro-apoptotic factors, such as Bax (Burlacu, 2003). Western blot analysis of lung lysates (Figure 4C) demonstrated that, unlike the increase in Bcl-2 expression, the expression of Bax was unaffected during chronic silicosis; thus, dramatically increasing the concentration of Bcl-2 in relation to Bax. Similarly, the concentration of Bax in BAL fluids was not significantly different between control, acute, and chronically silica-treated animals (not shown). Moreover, RT-PCR analysis of the lung RNA

indicated that the expression of Bcl-2 but not Bax was upregulated in the lungs from chronically silica-treated animals (not shown).

Bcl-2-positive Cells are Present within Chronic Silicotic Granulomas

Lung sections were examined for Bcl-2 expression by IHC to determine whether the increased expression of Bcl-2 protein in chronic silicosis is present in lung granuloma cells. Compared to the control lungs, the expression of Bcl-2 (brown staining) was significantly elevated within the lung granulomas from chronically (28 week) silica-exposed animals (Figure 5); however, the expression of Bcl-2 within the granulomas induced by acute silica exposure remained comparable to control lung tissues. The time course of immunohistological and granulomatous changes in the lung (data not shown) correlates well with the expression of Bcl-2 in BAL cells as determined by Western blot analysis (Figure 4A). Thus, Bcl-2 is expressed by BAL cells and the cells within chronic silicotic lung granulomas and may contribute to the lack of significant apoptosis in chronic silicosis.

NF- κ B-p50 Increases During Chronic Silicosis

Mounting evidence suggests that NF- κ B regulates induction of *Bcl-2* and the anti-apoptotic response (Catz and Johnson, 2001; Viatour et al., 2003), and silica was shown to increase NF- κ B expression in vitro (Kang et al., 2000) and in vivo (Hubbard et al., 2002). To examine the role of NF- κ B in chronic silicosis, the expression of immunoreactive NF- κ B-p50 and -p65 subunits was determined in lung sections from acutely and chronically silica-exposed animals by IHC. The anti-p50 antibody also reacts with the precursor form of NF- κ B-p50 - NF- κ B-p105. Compared to control lungs (Figure 6A), p50/p105 immunoreactivity (brown staining) is significantly increased in chronic (Figure 6B), but only moderately in acute silicotic granulomas (Figure 6C). On the other hand, the expression of the p65 subunit was comparable between the lungs from control (Figure 6D), chronic silicosis (Figure 6E), and acute silicosis (Figure 6F) animals. To initiate the transcription of NF- κ B regulated genes, the p50 and p65 subunits of NF- κ B translocate from the cytoplasm to the nucleus (Karin and Lin, 2002). The results in Figure 6B suggested that in chronic silicosis expression of p50/p105 was elevated both in the nucleus and the cytoplasm. To confirm the total rise of p50/p105 found by IHC as well as to determine whether the increased immunoreactivity of p50/p105 in chronic silicotic granulomas represents the accumulation of the inactive precursor (p105) or the active subunit (p50), the two proteins were resolved from the lung cell extracts by Western blot analysis. Figure 7A and 7B show that in chronic silicosis p50 expression is quantitatively higher than p105 and significantly higher than acute silicosis. Thus, chronic silicosis upregulates the expression of NF- κ B-p50 but not the p65 subunit. While the mechanism of selective accumulation of NF- κ B-p50 in chronic silicosis is not clear, it is possible that the overabundance of NF- κ B-p50 encourages the formation of transcriptionally inactive p50-p50 homodimers in chronic silicosis.

Bcl-3 Expression is Upregulated in Chronic Silicotic Granulomas

Bcl-3 is an I κ B-like protein that contains a transactivation domain that binds to p50-p50 homodimers, but not to p50-p65 heterodimers, and promotes the transcription of *Bcl-2* (Viatour et al., 2003). Figure 8 shows the IHC staining for Bcl-3 in the lung sections from control, and chronically and acutely silica-exposed animals. The results suggest that compared to control and acute silicotic lungs, chronic silicotic lungs contain a high concentration of Bcl-3 that is primarily localized to the nucleus of granulomatous epithelioid macrophages/multinucleated giant cells. Although not proven, the increased nuclear localization of Bcl-3 and NF- κ B-p50 might promote transcription of Bcl-2 in chronic silicotic granulomas.

Chronic Silicosis does not Alter Protein Ubiquitination

The accumulation of many proteins, such as procaspase-3, Bcl-2, and NF- κ B-p50, within chronic silicotic granulomas might result from silica-related damage of these proteins, leading to increased protein ubiquitination and overloading of the proteasome (Ardley and Robinson, 2004). However, Western blot analysis indicated that there were no significant differences in protein ubiquitination in the lungs from control, acute, and chronically silica-treated animals (data not shown). Thus, changes in protein ubiquitination are unlikely to account for increased buildup of several proteins in chronic silicosis.

Discussion

A notion, inferred primarily from acute high-dose silica exposure of animals or cell cultures, exists that apoptosis and lung inflammation are critical to the development of silicosis (Borges et al., 2001; 2002; Castranova et al., 2002; Porter et al., 2002; Santarelli et al., 2004). Other investigators and our group (Langley et al., 2004; Porter et al., 2006) demonstrated that rats exposed chronically to occupationally relevant doses of silica inhalation develop lung granulomas many months after silica exposure, and in the presence of only mild lung inflammation (Langley et al., 2004). Given that human silicosis remains asymptomatic for decades after silica exposure (Kreiss and Zhen, 1996), it is unlikely that it would remain clinically silent if it were accompanied with significant lung inflammation and tissue injury during early stages of the disease. Although the BAL fluids from chronically silica-exposed rats contain increased protein levels and lactate dehydrogenase (LDH) activity in the later stages of the disease (Langley et al., 2004), this could result primarily from endothelial leakage, necrotic death of some multinucleated giant cells, and/or degradation of extracellular matrix protein by metalloproteinases (unpublished observation) rather than true apoptotic cell death within the lung granulomas. Moreover, earlier reports also suggest that in chronically silica-exposed animals, lung injury continued to progress at later times although no significant damage was found immediately following the silica exposure (Porter et al., 2004; 2006).

The role of apoptosis in human granulomatous diseases is not clear, and whether apoptosis is beneficial or deleterious in granulomatous lung diseases is equivocal (Agostini et al., 1998; Budinger et al., 2006; Rossi et al., 2006). In animals models of acute silicosis, apoptosis is a critical event and Fas ligand-deficient mice do not develop acute silicosis in response to intratracheal deposition of large amounts of silica (Borges et al., 2001). Similarly, acute intratracheal deposition of bleomycin produces profound lung fibrosis within 3 weeks in wild-type mice but not in mice deficient in pro-apoptotic Bid protein (Budinger et al., 2006). However, little evidence supports the assertion that apoptosis is an important factor in chronic silicosis, the most prevalent form of silicosis in humans.

It is possible that apoptotic lung injury (i.e., presence of TUNEL positive cells) may have occurred during the early stages of chronic silica inhalation (i.e., during the 6 week silica exposure); however, significant lung injury during early stages of chronic granuloma formation was not observed (Langley et al., 2004). It is believed that decreased apoptosis might promote granuloma formation through accumulation of cells at the site of developing granuloma (Agostini et al., 1998). One of the concerns was that the lungs from neither control nor chronically silica-exposed animals exhibited significant TUNEL-positive cells, and might result from defective assay protocol/materials. Therefore, the competence of our apoptosis assays was ascertained by examining the lungs that were exposed to silica under established conditions for acute silicosis. Indeed, as noted by others (Borges et al., 2001), these lungs had significant number of TUNEL-positive and molecular imprints of apoptosis.

Apoptosis is regulated by a family of cysteine proteases (caspases) that are synthesized as inactive zymogens (pro-caspases) and require proteolytic cleavage by other (initiator) caspases

for activation (Boatright and Salvesen, 2003). Two major pathways regulate apoptosis: interaction of Fas with Fas ligand (Ashkenazi and Dixit, 1998) and disruption of mitochondrial integrity. The former activates caspase-8, and the latter releases cytochrome C from the mitochondria (Belka and Budach, 2002; Kadenbach et al., 2004), leading to activation of caspase-9. Caspase-3, the effector caspase in both the Fas- and mitochondrial-mediated apoptosis, is activated by the proteolytic cleavage of procaspase-3 by caspase-8 or caspase-9 (Boatright and Salvesen, 2003). Our results indicated that, unlike the lungs from animals with acute silicosis, which exhibit significantly higher level of caspase-3 activity, lungs from chronically silica-exposed animals had a significant buildup of procaspase-3 and virtually no silica-induced caspase-3 activity. The amount of procaspase-3 in the lungs of acutely silica-exposed animals was even lower than controls, regular increased protein loading to detect procaspase-3 band on the gels. The accumulation of various proteins, such as procaspase-3, Bcl-2; and NF- κ B-p50 in chronic silicosis was not related to a defective protein ubiquitination and degradation. It is, however not known, whether the buildup of procaspase-3 in chronic silicosis results from impaired activities of the upstream caspases that cleave procaspase-3 (i.e., caspases-8 and -9).

The activity of caspases is also regulated by the family of Bcl-2 proteins that include both pro- and anti-apoptotic proteins. While several of these family members affect apoptosis, the two important members of the family are the pro-apoptotic Bax and anti-apoptotic Bcl-2. These proteins promote and suppress caspase activation and apoptosis by increasing or preventing the release of mitochondrial cytochrome C, respectively (Er et al., 2006). Our results suggest that chronic silicosis promotes accumulation of the anti-apoptotic Bcl-2. Increasing evidence suggests that Bcl-2 not only affects the intrinsic (caspase 9-mediated) but also the extrinsic (caspase 8-mediated) apoptotic pathways (Nguyen et al., 2006; Sinicrope et al., 2004b). The reason for the buildup of Bcl-2 in BAL cells and lung granulomas from chronically silica-exposed animals is not clear; however, evidence suggests that a complex composed of Bcl-3 and NF- κ B-p50 homodimers acts as a potent transcription factor for *Bcl-2* (Bauer et al., 2006; Mitchell et al., 2001; Viatour et al., 2003). In this regard, our results show increased expression and nuclear localization of Bcl-3 and enhanced expression of NF- κ B-p50 relative to NF- κ B-p65 in chronic silicotic granulomas. This might encourage the formation of p50-p50 homodimers and their interaction with Bcl-3, promoting the transcription of *Bcl-2*. The mechanism for the accumulation of NF- κ B-p50 without a similar increase in NF- κ B-p105 is not clear at present and may be related to the chronic effects of silica inhalation. In addition, while at present, it is not possible to attribute the increased Bcl-2 expression in chronic silicosis to the elevated levels and nuclear localization of Bcl-3 and NF- κ B-p50; however, the presence of essentially all Bcl-3 in the nucleus makes it a good possibility and requires further investigation. Moreover, unlike the NF κ B-p50-p65 complex (Laza-Stanca et al., 2006; Vanden Berghe et al., 2003), the Bcl-3-p50-p50 complex downregulates the transcription of pro-inflammatory cytokine genes such as TNF- α (Kuwata et al., 2003), and our preliminary genomic studies (Langley et al., Personal Communication) suggest that, unlike acute silicosis, chronic silicosis is not associated with highly increased expression of TNF- α or IL-1 β . Similarly, while acute exposure of animals and cell cultures to high concentrations of asbestos induces pro-apoptotic cytokines such as TNF- α and IL-1 β (Li et al., 1993; Zhang and Phan, 1996), BAL fluids from asbestos-exposed workers do not contain high levels of these cytokines (Mutsaers et al., 1998).

In animal models of acute silicosis, silica increases Fas expression and apoptosis (Borges et al., 2001); however, little evidence supports a significant role of apoptosis in the development of human chronic silicosis. In fact, the presence of elevated levels of the anti-apoptotic protein soluble Fas in the serum and its message in peripheral blood mononuclear cells from silicosis patients (Otsuki et al., 2000) indicates that the granuloma formation in human silicosis may not be associated with apoptotic cell death. The presence of anti-apoptotic factors, including

soluble Fas and soluble Fas ligand, was also found in other fibrotic/granulomatous human lung diseases, including tuberculosis, sarcoidosis, and pulmonary fibrosis (Kuwano et al., 2000; Ordway et al., 2005; Shikuwa et al., 2002). Moreover, the results from other proliferative diseases suggest that apoptosis is associated with improvement in the disease. Thus, reduction in colorectal adenomas in response to celecoxib is related to its pro-apoptotic properties (Sinicrope et al., 2004a). Similarly, the anti-tumorigenic properties and anti-fibrotic effects of curcumin are linked to its pro-apoptotic properties (Agarwal et al., 2002). Apoptosis may also be more closely associated with resolution rather than generation of inflammation and fibrosis in these diseases (Agostini et al., 1998; Cox et al., 1995; Haslett, 1992). Thus, in pulmonary sarcoidosis, the presence of TUNEL-positive cells within lung granulomas suggests a better prognosis (Kunitake et al., 1999), and drug-induced apoptosis ameliorates bleomycin-induced lung injury and carrageenan-elicited acute pleurisy (Rossi et al., 2006). On the other hand, expression of anti-apoptotic markers within the foamy macrophages of lung granulomas in *Mycobacterium tuberculosis*-infected mice is associated with the progression and exacerbation of the granulomatous disease (Ordway et al., 2005). Thus, apoptosis is an important component of acute lung injury; however, chronic inflammatory granulomatous diseases might result from the accumulation of cells that are relatively resistant to apoptotic death.

In addition to difference in the apoptotic response, acute and chronic silicotic granulomas also differ in histology. The acute silicotic lung granulomas in rats consist of loosely aggregated, activated, foamy histiocytes intermixed with lymphocytes. On the other hand, the granulomas from chronically silica-exposed animals are discrete and well organized, and composed primarily of epithelioid macrophages and multinucleated giant cells (Chambers, 1978), and are surrounded by lymphocytes and neutrophils (classic granulomas). Multinucleated giant cells are seen in many human lung diseases resulting from occupational exposure to inorganic particles, including silica (Mancino and Caselli, 1966; Takemura et al., 1989).

Thus our results suggest that unlike acute silicosis, chronic silicosis is associated with limited inflammation and an anti-apoptotic response. This might explain the resistance of human silicosis to anti-inflammatory therapies. On the other hand, it is plausible that activation of apoptosis by more potent and novel pro-apoptotic drugs might ameliorate chronic granulomatous lung diseases, including chronic silicotic granulomas.

Acknowledgments

We thank Drs. Chien-An Hu and Scott Burchiel for their helpful suggestions. We also thank Vicki Fisher for editorial help and Steve Randock for graphics. All animal studies were conducted at Lovelace Respiratory Research Institute, a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

References

- Agarwal B, Halmos B, Feoktistov AS, Protiva P, Ramey WG, Chen M, Pothoulakis C, Lamont JT, Holt PR. Mechanism of lovastatin-induced apoptosis in intestinal epithelial cells. *Carcinogenesis* 2002;23:521–528. [PubMed: 11895868]
- Agostini C, Perin A, Semenzato G. Cell apoptosis and granulomatous lung diseases. *Curr Opin Pulm Med* 1998;4:261–266. [PubMed: 10813200]
- Ardley HC, Robinson PA. The role of ubiquitin-protein ligases in neurodegenerative disease. *Neurodegener Dis* 2004;1:71–87. [PubMed: 16908979]
- Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998;281:1305–1308. [PubMed: 9721089]
- Barbarin V, Xing Z, Delos M, Lison D, Huaux F. Pulmonary overexpression of IL-10 augments lung fibrosis and Th2 responses induced by silica particles. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L841–L848. [PubMed: 15608148]

- Barbaro M, Cutroneo G, Costa C, Sciorio S, Trimarchi F, Favaloro A, Fenga C, Barbaro ML, Spataro G, Abbate C, Bramanti P. Early events of experimental exposure to amorphous and crystalline silica in the rat: time course of surfactant protein D. *Ital J Anat Embryol* 2002;107:243–256. [PubMed: 12611476]
- Bauer A, Villunger A, Labi V, Fischer SF, Strasser A, Wagner H, Schmid RM, Hacker G. The NF-kappaB regulator Bcl-3 and the BH3-only proteins Bim and Puma control the death of activated T cells. *Proc Natl Acad Sci USA* 2006;103:10979–10984. [PubMed: 16832056]
- Belka C, Budach W. Anti-apoptotic Bcl-2 proteins: structure, function and relevance for radiation biology. *Int J Radiat Biol* 2002;78:643–658. [PubMed: 12194748]
- Boatright KM, Salvesen GS. Mechanisms of caspase activation. *Curr Opin Cell Biol* 2003;15:725–731. [PubMed: 14644197]
- Borges VM, Falcao H, Leite-Junior JH, Alvim L, Teixeira GP, Russo M, Nobrega AF, Lopes MF, Rocco PM, Davidson WF, Linden R, Yagita H, Zin WA, DosReis GA. Fas ligand triggers pulmonary silicosis. *J Exp Med* 2001;194:155–164. [PubMed: 11457890]
- Borges VM, Lopes MF, Falcao H, Leite-Junior JH, Rocco PR, Davidson WF, Linden R, Zin WA, DosReis GA. Apoptosis underlies immunopathogenic mechanisms in acute silicosis. *Am J Respir Cell Mol Biol* 2002;27:78–84. [PubMed: 12091249]
- Budinger GR, Mutlu GM, Eisenbart J, Fuller AC, Bellmeyer AA, Baker CM, Wilson M, Ridge K, Barrett TA, Lee VY, Chandel NS. Proapoptotic Bid is required for pulmonary fibrosis. *Proc Natl Acad Sci USA* 2006;103:4604–4609. [PubMed: 16537427]
- Burlacu A. Regulation of apoptosis by Bcl-2 family proteins. *J Cell Mol Med* 2003;7:249–257. [PubMed: 14594549]
- Castranova V, Porter D, Millecchia L, Ma JY, Hubbs AF, Teass A. Effect of inhaled crystalline silica in a rat model: time course of pulmonary reactions. *Mol Cell Biochem* 2002;234–235. 177–184.
- Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. *Oncogene* 2001;20:7342–7351. [PubMed: 11704864]
- Chambers TJ. Multinucleate giant cells. *J Pathol* 1978;126:125–148. [PubMed: 370353]
- Chong S, Lee KS, Chung MJ, Han J, Kwon OJ, Kim TS. Pneumoconiosis: comparison of imaging and pathologic findings. *Radiographics* 2006;26:59–77. [PubMed: 16418244]
- Cox G, Crossley J, Xing Z. Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. *Am J Respir Cell Mol Biol* 1995;12:232–237. [PubMed: 7865221]
- Er E, Oliver L, Cartron PF, Juin P, Manon S, Vallette FM. Mitochondria as the target of the pro-apoptotic protein Bax. *Biochim Biophys Acta* 2006;1757:1301–1311. [PubMed: 16836974]
- Haslett C. Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. *Clin Sci (Lond)* 1992;83:639–648. [PubMed: 1336433]
- Hnizdo E, Sluis-Cremer GK. Risk of silicosis in a cohort of white South African gold miners. *Am J Ind Med* 1993;24:447–457. [PubMed: 8250063]
- Hnizdo E, Vallyathan V. Chronic obstructive pulmonary disease due to occupational exposure to silica dust: a review of epidemiological and pathological evidence. *Occup Environ Med* 2003;60:237–243. [PubMed: 12660371]
- Hubbard AK, Timblin CR, Shukla A, Rincon M, Mossman BT. Activation of NF-kappaB-dependent gene expression by silica in lungs of luciferase reporter mice. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L968–L975. [PubMed: 11943661]
- Hughes JM, Weill H, Checkoway H, Jones RN, Henry MM, Heyer NJ, Seixas NS, Demers PA. Radiographic evidence of silicosis risk in the diatomaceous earth industry. *Am J Respir Crit Care Med* 1998;158:807–814. [PubMed: 9731009]
- Kadenbach B, Arnold S, Lee I, Huttemann M. The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases. *Biochim Biophys Acta* 2004;1655:400–408. [PubMed: 15100056]
- Kang JL, Go YH, Hur KC, Castranova V. Silica-induced nuclear factor-kappaB activation: involvement of reactive oxygen species and protein tyrosine kinase activation. *J Toxicol Environ Health A* 2000;60:27–46. [PubMed: 10832616]

- Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol* 2002;3:221–227. [PubMed: 11875461]
- Kreiss K, Zhen B. Risk of silicosis in a Colorado mining community. *Am J Ind Med* 1996;30:529–539. [PubMed: 8909602]
- Kunitake R, Kuwano K, Miyazaki H, Hagimoto N, Nomoto Y, Hara N. Apoptosis in the course of granulomatous inflammation in pulmonary sarcoidosis. *Eur Respir J* 1999;13:1329–1337. [PubMed: 10445608]
- Kuwano K, Kawasaki M, Maeyama T, Hagimoto N, Nakamura N, Shirakawa K, Hara N. Soluble form of fas and fas ligand in BAL fluid from patients with pulmonary fibrosis and bronchiolitis obliterans organizing pneumonia. *Chest* 2000;118:451–458. [PubMed: 10936140]
- Kuwata H, Watanabe Y, Miyoshi H, Yamamoto M, Kaisho T, Takeda K, Akira S. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-alpha production in macrophages. *Blood* 2003;102:4123–4129. [PubMed: 12907458]
- Langley RJ, Kalra R, Mishra NC, Hahn FF, Razani-Boroujerdi S, Singh SP, Benson JM, Pena-Philippides JC, Barr EB, Sopori ML. A biphasic response to silica: I. Immunostimulation is restricted to the early stage of silicosis in Lewis rats. *Am J Respir Cell Mol Biol* 2004;30:823–829. [PubMed: 14742293]
- Laza-Stanca V, Stanciu LA, Message SD, Edwards MR, Gern JE, Johnston SL. Rhinovirus replication in human macrophages induces NF-kappaB-dependent tumor necrosis factor alpha production. *J Virol* 2006;80:8248–8258. [PubMed: 16873280]
- Li XY, Lamb D, Donaldson K. The production of TNF-alpha and IL-1-like activity by bronchoalveolar leucocytes after intratracheal instillation of crocidolite asbestos. *Int J Exp Pathol* 1993;74:403–410. [PubMed: 8398814]
- Mancino D, Caselli P. Granulomatous reactions induced by silica. I Relationships between particles size and multinucleated giant cells. *Folia Med (Napoli)* 1966;49:85–121. [PubMed: 4287453]
- McLaughlin JK, Chow WH, Levy LS. Amorphous silica: a review of health effects from inhalation exposure with particular reference to cancer. *J Toxicol Environ Health* 1997;50:553–66. [PubMed: 15279029]
- Mitchell TC, Hildeman D, Kedl RM, Teague TK, Schaefer BC, White J, Zhu Y, Kappler J, Marrack P. Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat Immunol* 2001;2:397–402. [PubMed: 11323692]
- Mutsaers SE, Harrison NK, McAnulty RJ, Liao JY, Laurent GJ, Musk AW. Fibroblast mitogens in bronchoalveolar lavage (BAL) fluid from asbestos-exposed subjects with and without clinical evidence of asbestosis: no evidence for the role of PDGF, TNF-alpha, IGF-1, or IL-1 beta. *J Pathol* 1998;185:199–203. [PubMed: 9713348]
- Nguyen DM, Yeow WS, Ziauddin MF, Baras A, Tsai W, Reddy RM, Chua A, Cole GW Jr, Schrupp DS. The essential role of the mitochondria-dependent death-signaling cascade in chemotherapy-induced potentiation of Apo2L/TRAIL cytotoxicity in cultured thoracic cancer cells: amplified caspase 8 is indispensable for combination-mediated massive cell death. *Cancer J* 2006;12:257–273. [PubMed: 16925970]
- Ordway D, Henao-Tamayo M, Orme IM, Gonzalez-Juarrero M. Foamy macrophages within lung granulomas of mice infected with *Mycobacterium tuberculosis* express molecules characteristic of dendritic cells and antiapoptotic markers of the TNF receptor-associated factor family. *J Immunol* 2005;175:3873–3881. [PubMed: 16148133]
- Otsuki T, Sakaguchi H, Tomokuni A, Aikoh T, Matsuki T, Isozaki Y, Hyodoh F, Kawakami Y, Kusaka M, Kita S, Ueki A. Detection of alternatively spliced variant messages of Fas gene and mutational screening of Fas and Fas ligand coding regions in peripheral blood mononuclear cells derived from silicosis patients. *Immunol Lett* 2000;72:137–143. [PubMed: 10841950]
- Poljakovic M, Porter DW, Millecchia L, Kepka-Lenhart D, Beighley C, Wolfarth MG, Castranova V, Morris SM Jr. Cell- and isoform-specific increases in arginase expression in acute silica-induced pulmonary inflammation. *J Toxicol Environ Health A* 2007;70:118–127. [PubMed: 17365572]
- Porter DW, Barger M, Robinson VA, Leonard SS, Landsittel D, Castranova V. Comparison of low doses of aged and freshly fractured silica on pulmonary inflammation and damage in the rat. *Toxicology* 2002;175:63–71. [PubMed: 12049836]

- Porter DW, Hubbs AF, Mercer R, Robinson VA, Ramsey D, McLaurin J, Khan A, Battelli L, Brumbaugh K, Teass A, Castranova V. Progression of lung inflammation and damage in rats after cessation of silica inhalation. *Toxicol Sci* 2004;79:370–380. [PubMed: 15056817]
- Porter DW, Millecchia LL, Willard P, Robinson VA, Ramsey D, McLaurin J, Khan A, Brumbaugh K, Beighley CM, Teass A, Castranova V. Nitric oxide and reactive oxygen species production causes progressive damage in rats after cessation of silica inhalation. *Toxicol Sci* 2006;90:188–197. [PubMed: 16339787]
- Razani-Boroujerdi S, Sopor ML. Early manifestations of NNK-induced lung cancer: role of lung immunity in tumor susceptibility. *Am J Respir Cell Mol Biol* 2007;36:13–19. [PubMed: 16873770]
- Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, Caldicott A, Martinez-Losa M, Walker TR, Duffin R, Gray M, Crescenzi E, Martin MC, Brady HJ, Savill JS, Dransfield I, Haslett C. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. *Nat Med* 2006;12:1056–1064. [PubMed: 16951685]
- Santarelli L, Recchioni R, Moroni F, Marcheselli F, Governa M. Crystalline silica induces apoptosis in human endothelial cells in vitro. *Cell Biol Toxicol* 2004;20:97–108. [PubMed: 15242185]
- Shi X, Castranova V, Halliwell B, Vallyathan V. Reactive oxygen species and silica-induced carcinogenesis. *J Toxicol Environ Health B* 1998;1:181–97.
- Shikuwa C, Kadota J, Mukae H, Iwashita T, Kaida H, Ishii H, Ishimatsu Y, Kohno S. High concentrations of soluble Fas ligand in bronchoalveolar lavage fluid of patients with pulmonary sarcoidosis. *Respiration* 2002;69:242–246. [PubMed: 12097768]
- Sinicrope FA, Half E, Morris JS, Lynch PM, Morrow JD, Levin B, Hawk ET, Cohen DS, Ayers GD, Stephens LC. Cell proliferation and apoptotic indices predict adenoma regression in a placebo-controlled trial of celecoxib in familial adenomatous polyposis patients. *Cancer Epidemiol Biomarkers Prev* 2004a;13:920–927. [PubMed: 15184247]
- Sinicrope FA, Penington RC, Tang XM. Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis is inhibited by Bcl-2 but restored by the small molecule Bcl-2 inhibitor, HA 14-1, in human colon cancer cells. *Clin Cancer Res* 2004b;10:8284–8292. [PubMed: 15623604]
- Takemura T, Rom WN, Ferrans VJ, Crystal RG. Morphologic characterization of alveolar macrophages from subjects with occupational exposure to inorganic particles. *Am Rev Respir Dis* 1989;140:1674–1685. [PubMed: 2557785]
- Tomokuni A, Aikoh T, Matsuki T, Isozaki Y, Otsuki T, Kita S, Ueki H, Kusaka M, Kishimoto T, Ueki A. Elevated soluble Fas/APO-1 (CD95) levels in silicosis patients without clinical symptoms of autoimmune diseases or malignant tumours. *Clin Exp Immunol* 1997;110:303–309. [PubMed: 9367417]
- Vallyathan V, Green F, Ducatman B, Schulte P. Roles of epidemiology, pathology, molecular biology, and biomarkers in the investigation of occupational lung cancer. *J Toxicol Environ Health B* 1998;1:91–116.
- Vanden Berghe W, Vermeulen L, Delerive P, De Bosscher K, Staels B, Haegeman G. A paradigm for gene regulation: inflammation, NF-kappaB and PPAR. *Adv Exp Med Biol* 2003;544:181–196. [PubMed: 14713228]
- Viatour P, Bentires-Alj M, Chariot A, Deregowski V, de Leval L, Merville MP, Bours V. NF- kappa B2/p100 induces Bcl-2 expression. *Leukemia* 2003;17:1349–1356. [PubMed: 12835724]
- Williams GT, Williams WJ. Granulomatous inflammation--a review. *J Clin Pathol* 1983;36:723–733. [PubMed: 6345591]
- Zhang DD, Hartsy MA, Warheit DB. Time course of quartz and TiO(2) particle-induced pulmonary inflammation and neutrophil apoptotic responses in rats. *Exp Lung Res* 2002;28:641–670. [PubMed: 12490038]
- Zhang K, Phan SH. Cytokines and pulmonary fibrosis. *Biol Signals* 1996;5:232–239. [PubMed: 8891199]

Figure 1. Acute but not chronic silicosis is associated with apoptosis

(A) Tissue sections from control (left), chronic (center), and acute (right) silicotic lungs were examined by TUNEL assay. Animals chronically and acutely exposed to silica were sacrificed at week 28 and day 14 after silica exposure, respectively. TUNEL-positive cells (nuclear condensates) present in the lung sections from acutely exposed animals are indicated by arrows and magnified in the inset. The figure is a representative of 5 animals per group. (B) Quantitative analysis of TUNEL activity for apoptotic cells at week 28 chronic silica exposure and day 14 acute silica exposure is represented as the number of nuclei that have apoptotic bodies (nuclear condensates) found within a field of granulomatous inflammatory sites (3 microscopic fields per animal/5 animals per group). No apoptotic bodies were found at day 4 or week 7 and 14 (data not shown). * Significant from $p < 0.05$.

Figure 2. BAL cells from animals chronically exposed to silica show anti-apoptotic markers
BAL cell lysates examined by Western blot analysis show the following: (A) procaspase 3 content at 14 week post chronic silica inhalation; (B) procaspase 3 content at day 14 after acute silica instillation. Loading controls were determined by GAPDH. Significantly more protein was loaded in the acute samples (100 μ g) as was loaded in the chronic samples (10 μ g) – thus the marked group differences in the amount of control procaspase 3 as well as total GAPDH. Data is representative of 5 animals per group (C, D) densitometry readings were normalized with GAPDH and were calculated from 5 animals per group and is represented as a change in % of control (* $p < 0.05$).

Figure 3. Acute silicosis increases caspase-3 activity in the lung

Caspase-3 activity of lung lysates was determined by ELISA as described in the Methods section. Each bar graph represents the enzyme activity $\pm$ SE of 3 different lung samples and the assay was run in duplicates at two different dilutions. Statistical significance was analyzed by GraphPad Prism.

Figure 4. Bcl-2 but not Bax is upregulated in chronic silicosis

(A) Changes in Bcl-2 expression by Western blots in BAL cell lysates at the indicated times after chronic silica exposure. Each lane is from an individual animal at the time point. Loading controls determined by GAPDH expression. (B) Densitometry of Figure 3A at day 4 or week 7, 14, and 28 after silica exposure. The readings were normalized with GAPDH and presented as a change in % of control. (C) Western blot analysis of lung cell lysates for the presence of Bcl-2 and Bax at 28 weeks, each lane represents an individual animal (* $p < 0.05$).

6314-4

Figure 5. Bcl-2 expression is upregulated in lung granulomas from chronically silica-exposed animals

Lung sections from control (left), chronic 28 week (center), and acute 14 day (right) silicotic lungs were examined by IHC staining for Bcl-2 expression as described in Methods. The majority of stain was found within the epitheloid macrophages. Data represent 5 animals per group.

6314-5

Figure 6. Chronic silicosis increases p50 but not p65 immunoreactivity in the lung

Lung sections from control, chronic 28 week, and acute 14 day silicotic lungs were analyzed by IHC as described in Methods. Upper panels (A–C) show p50, and lower panels (D–F) show p65 reactivity. The figures are representative of 5 animals per group.

Figure 7. Chronic silicosis increases p50 immunoreactivity in the lung

(A) Cell extracts made from control, chronic week 28, and acute day 14 silicotic lungs were examined by Western blot analysis as described in Methods. Data is representative of 5 animals per group and GAPDH was performed to verify proper loading. (B) Densitometry for p105, p50, and p65 was normalized with GAPDH and is presented as change % of control. * $p < 0.05$.

Figure 8. Bcl-3 is mainly localized to the nucleus in chronic silicotic granulomas

(A) Tissue sections from control, chronic week 28, and acute day 14 silicotic lungs were examined for Bcl-3 expression by IHC as described in Methods. The figure represents data from 5 animals per group. (B) Bcl-3 staining. Cells were enumerated by counting the number of nuclei that stained positive for Bcl-3 (suggesting nuclear localization) within a microscopic field of granulomatous inflammation. Three fields per animal, 5 animals per group. * $p < 0.05$. In control animals, other than occasional alveolar macrophages that demonstrated nuclear localization the vast majority of Bcl-3 staining did not appear localized to the nucleus (data not shown).