Localization of Intercellular Adhesion Molecule-1 (ICAM-1) in the Lungs of Silica-exposed Mice

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Intercellular adhesion molecule-1 (ICAM-1) is expressed on a variety of cells including endothelial cells, alveolar epithelial cells, and alveolar macrophages. Endothelial/epithelial cell ICAM-1 participates in the migration of leukocytes out of the blood in response to pulmonary inflammation, whereas alveolar macrophage ICAM-1 may represent cell activation. Our previous studies have shown that there is increased expression of ICAM-1 in lung tissue during acute inflammation following intratracheal injection with silica particles (2 mg/mouse). This increased expression was shown to play a role, in part, in the migration of neutrophils from the circulation into the tissue parenchyma. The aim of the current work is to localize expression of ICAM-1 during acute inflammation in lungs of mice exposed to either silica or the nuisance dust, titanium dioxide. In silica-exposed mice, a significant increase in ICAM-1 was detected on day 1 and localized by immunohistochemistry to aggregates of pulmonary macrophages and to type II epithelial cells. Areas of the lung with increased ICAM-1 expression also showed increased tumor necrosis factor alpha expression. Immunocytotoxic staining of bronchoalveolar lavage (BAL) cells demonstrated increased ICAM-1 expression associated with alveolar macrophages 3, 5, and 7 days following silica exposure. Finally, soluble ICAM-1 levels in the BAL fluid were significantly increased in mice exposed to silica on the same days. Titanium dioxide exposure elicited a minimal increase in expression of ICAM-1 in the lungs. These data demonstrate that exposure to the toxic particle silica specifically increases ICAM-1 expression localized to pulmonary macrophages and type II epithelial cells. — Environ Health Perspect 105(Suppl 5):1183–1190 (1997)

Key words: ICAM-1, adhesion molecules, alveolar macrophages, pulmonary inflammation, silica, titanium dioxide

Introduction

Exposure of mice to silica particles (\(\alpha\)-quartz) generates an intense inflammatory response characterized by the influx of neutrophils and monocytes into the alveoli (1–3). Phagocytosis of these silica particles by inflammatory cells may result in damage to the lung epithelium through the generation of free radicals (4–7) and the release of lysosomal enzymes (8). In response to injury, activated macrophages release fibrogenic factors (2,9,10), which leads to pulmonary fibrosis (11).

The mechanism by which these inflammatory cells are recruited to specific sites in the lungs is a complex process involving the up- and downregulation of adhesion molecules on the leukocytes as well as on lung cells in areas of pulmonary inflammation. These molecules are membrane-bound proteins that function in cell-to-cell interactions, including the migration of cells from the blood through the pulmonary interstitium and into the alveolar space (12–14). One such adhesion protein, intercellular adhesion molecule-1 (ICAM-1), is expressed on a variety of cells including endothelial cells, fibroblasts, types I and II alveolar epithelial cells, and monocyte/macrophages (15). The cytokines, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-1 (IL-1), and interferon gamma (IFN-\(\gamma\)) upregulate ICAM-1 expression on alveolar macrophages (AM), endothelial cells, and lung epithelial cells (15–17). ICAM-1 is capable of binding to the \(\beta\)-2 integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), both of which are expressed on neutrophils (18,19). Through this interaction, it is hypothesized that neutrophils migrate to areas of inflammation from the circulation (20).

In addition to the membrane-bound form of ICAM-1, a circulating form of this protein has been described in human serum (21). Although the function and source of soluble ICAM-1 (sICAM-1) are still under investigation, elevated levels of this protein have been detected in the sera of patients with idiopathic pulmonary fibrosis (22) and the bronchoalveolar lavage (BAL) fluid of rats with fibrotic lung disease (23).

Previous work from this laboratory documented that silica exposure increased the amount of ICAM-1 protein in homogenized lungs from mice (24). The present experiments were conducted to localize the expression of ICAM-1 over the first 7 days following silica exposure and to compare the response between mice exposed to silica and mice exposed to the nuisance dust, titanium dioxide.

Materials and Methods

Animals

Adult female C57BL/6 mice (9–15 weeks of age; Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Animals were housed five per cage under horizontal laminar air flow and maintained on a 12-hr light/12-hr dark cycle in a temperature-controlled animal care facility. Food and tap water were available ad libitum throughout all studies.

Instillation of Particles

Silica crystals (\(\alpha\)-quartz; Min-U-Sil 5; \(<5.0 \mu m\); Pennsylvania Glass and Sand, Pittsburgh, PA) or titanium dioxide (5.0 \(\mu m\); Sigma Chemical, St. Louis, MO) were administered as previously described (7). Briefly, mice were anesthetized by an ip injection of sodium pentobarbital (40 mg/kg) and their tracheas exposed by dissection. Two milligrams of silica or titanium dioxide was injected intratracheally in two bolus doses of 1 mg in 0.05 ml sterile saline. Titanium dioxide served as a low toxicity particle control and sterile saline...
served as a vehicle control. Both the titanium dioxide and saline were also administered in two bolus doses, each in 0.05-ml volumes, similar to the silica injection.

**Bronchoalveolar Lavage**

Mice were euthanized by an overdose of sodium pentobarbital ip at different times after particle or saline exposure. Lungs were lavaged in situ three times with 1 ml sterile saline each; lavage returns of 0.95 ml were similar among all treatment groups. All lavages were centrifuged at 250 × g for 10 min at 4°C. The cell-free supernatants from the first two lavages were pooled and frozen at −80°C for later evaluation of sICAM-1 levels. The cell pellets from all three lavages were pooled and assessed for cell number and type. Cells were resuspended in 1% bovine serum albumin in saline and centrifuged onto a glass slide using a Shandon cytocentrifuge (Shandon Southern Instruments, Sewickley, PA). Specimens were fixed in acetone at 4°C and stored at −20°C.

**Measurement of Soluble ICAM-1**

Soluble ICAM-1 levels were measured by an antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Endogen, Cambridge, MA). BAL fluid samples were diluted 1:50. Fifty microliters of sample was incubated on antibody precoated wells. After 2 hr, a labeled antibody directed against sICAM-1 was added. After addition of substrate, absorbance was read at 450 nm. Simple linear regression was performed using sICAM-1 protein as a standard and data expressed as nanograms of sICAM-1/ml recovered BAL fluid.

**Isolation of Lung Tissue Protein**

Lung protein was isolated according to Syrbu et al. (25). After BAL, the lungs were perfused through the right ventricle with sterile phosphate-buffered saline (PBS), pH 7.0. The lungs were excised en bloc and all surrounding tissue removed. The lungs were then minced in ice-cold homogenization buffer (1 mM EGTA, 1 mM PMSF, 1.5 μM peptatin, 2 μM leupeptin, 1 μM benzamidine, and 1 μg/ml aprotonin in 20 mM Tris-maleate buffer, pH 7.0). The homogenate was centrifuged at 11,000 × g for 10 min at 4°C. The supernatants were collected and centrifuged again at 225,000 × g for 16 min at 4°C. The resulting pellets were then resuspended using a Dounce homogenizer in the homogenization buffer. Aliquots were frozen at −80°C until their evaluation for levels of ICAM-1 protein.

**Enzyme-linked Immunosorbent Assay for Lung Tissue ICAM-1**

This assay is a modification of that described by Syrbu et al. (25). Five milligrams of lung protein in 100 μl of 0.5-M carbonate buffer, pH 9.6, was incubated overnight at 4°C in a 96-well Immulon-2 (Dynatech Laboratories, Chantilly, VA) ELISA plate. The next day, the plate was washed three times with 0.05% tween 20 in PBS (TPBS), pH 8.2. The wells were then blocked using 0.2% TPBS for 1 hr at room temperature. The wells were washed 3× with TPBS, then the plate was incubated with 100 μl of anti-ICAM-1 antibody (KAT-1; Seikagaku America; Rockville, MD) for 2 hr at room temperature. IgG2b (PharMingen; San Diego, CA) was used as an isotype antibody control. The wells were washed 3× with TPBS, then incubated with peroxidase conjugated goat antirat antibody (Sigma Chemical) for 1 hr at room temperature. After a final wash (3×) with TPBS, the substrate ABTS and 10 μl H2O2 were added and the optical density at 410/490 nm read (Dynatech MicroELISA plate reader).

**Preparation of Parenchymal Lung Tissue**

Lungs were fixed by microwave heating (26). Briefly, lungs were inflated with ice-cold saline and the tracheas tied with nylon thread. The lungs were then placed in room temperature saline and microwaved for 5 min at 60°C, then placed in 50% ethanol. Lungs were embedded in paraffin and sectioned.

**Immunohistochemistry**

Both lavaged cells pelleted by cytopsin and fixed lungs processed for immunohistochemistry were assessed for ICAM-1 expression using the protocol described by the BioGenex Super Sensitive Detection System (BioGenex; San Ramon, CA). Briefly, endogenous peroxidase in specimens was inactivated by incubation with 3% H2O2 for 5 min. After two washes with PBS, the slides were incubated for 2 hr with YN1/1.7.4 antibody (anti-ICAM-1; gift of R. Rothlein, Boehringer Ingelheim; Ridgefield, CT) as the primary antibody. Alternately, serial lung sections were incubated with rat antimouse TNF-α (PharMingen). The negative control used for both antibodies was a nonspecific rat antibody provided by BioGenex. Following three washes with PBS, slides were incubated with Link, biotinylated goat antirat secondary antibody. After two washes with PBS, slides were incubated with Label, horseradish peroxidase conjugated streptavidin. After two more washes with PBS, slides were incubated with substrate and catalyst, amino-9-ethylcarbazole chromogen and H2O2. Counterstaining was performed using Mayer’s hematoxylin. Photomicrographs (>400, ×1000) were taken under light microscopy of representative fields. BAL cells were scored based on staining from 0 to 4 (0, no staining; 4, intense staining). The number of cells per score was multiplied by that score and totaled to represent the ICAM-1 immunostaining index.

**Statistics**

All data are expressed as mean ± SE of three to eleven mice for each exposure group for each time point. Multiple comparisons were made by two-factor analysis of variance followed by the Neuman–Keuls post-hoc test (Statistica Software, StatSoft; Tulsa, OK). p < 0.05 was required for statistical significance.

**Results**

**Silica-induced ICAM-1 Expression**

Instillation of silica particles significantly elevated lung tissue ICAM-1 levels 1 day after exposure as measured in homogenized lung tissue by ELISA (Figure 1). This expression evidenced by immunohistochemistry appeared to be localized to alveolar and interstitial macrophages as well as type II epithelial cells (Figure 2A, B). Neither

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**Figure 1.** sICAM-1 expression in mice lungs following particle exposure. Mice were exposed to 2 mg/mouse silica, titanium dioxide, or saline. At various times postinstillation, the animals were sacrificed and levels of ICAM-1 in homogenized lung tissue measured by ELISA. Data are expressed as fold increase above constitutive levels (those detected in naive mice). Each data point is the mean of three to eight mice ± SEM, p < 0.05 * significant different from saline-instilled mice; ** significant different from TiO2-instilled mice.
Figure 2. Detection of ICAM-1 in lung tissue. Mice were exposed to 2 mg/mouse (A, B) silica, (C) titanium dioxide, or (D) saline and sacrificed 1 day postinstillation. Brown areas indicate areas positive for ICAM-1 by staining with a monoclonal antibody specific for mouse ICAM-1. Photomicrographs are shown at A, C, and D × 400 and B × 1000.
Titanium dioxide nor saline instillation significantly elevated ICAM-1 levels as measured by ELISA (Figure 1) or by immunohistochemistry (Figure 2C, D). Deposition of titanium dioxide particles in the lungs was readily apparent. Exposure to silica also elicited a marked elevation in TNF-α expression, which appeared to be localized to the same histologic areas with increased ICAM-1 expression (Figure 3A, B).

AM lavaged from the lungs of silica-exposed mice also demonstrated increased expression of ICAM-1 (immunocytochemistry) that increased with time. Initially on days 1 and 3, silica elicited a modest expression of ICAM-1, detected primarily on the membranes of AM. However, 5 days after silica exposure, a dramatic increase in expression of ICAM-1 (Figure 4) was observed associated with both AM membranes and cytoplasm (Figure 5A). Neither titanium dioxide nor saline instillation elicited this degree of expression associated with AM (Figures 4, 5B, C). Again, titanium dioxide particles engulfed by AM were apparent.

sICAM-1 was measured in the BAL fluid of particle exposed mice. Significantly increased levels of sICAM-1 were found in the BAL fluid of mice exposed to silica (Figure 6) with times of increase (days 3, 5, and 7) parallel to increased AM-associated ICAM-1. Titanium dioxide exposure also elicited increased levels of sICAM-1, but well below that observed in silica exposed animals. Neither particle increased levels of circulating (plasma) sICAM-1 (data not shown). Thus, increased levels of ICAM-1 in lung tissue associated with AM, and in the BAL fluid, were the result of exposure to the toxic particle, silica, but not to the nuisance dust, titanium dioxide.

Silica-induced Pulmonary Inflammation

Studies were next conducted to examine cell influx into the airways following exposure to silica or titanium dioxide and the

Figure 3. Detection of ICAM-1 and TNF-α in serial lung tissue sections. Mice were exposed to 2 mg/mouse silica and sacrificed 1 day later. (A) Detection of TNF-α expression appears to be in the same histologic areas as (B) detection of ICAM-1 expression. Photomicrographs are shown at × 400.

Figure 4. Immunostaining index of alveolar macrophage-associated ICAM-1. Mice were exposed to 2 mg/mouse silica, titanium dioxide, or saline and sacrificed at various times postinstillation. BAL cells were scored based on staining from 0 to 4 (0, no staining; 4, intense staining). The number of cells per score was multiplied by that score and totaled to represent the immunostaining index. Each data point for silica- and titanium dioxide-exposed mice is the mean of three to four animals ± SEM, p ≤ 0.05. +, significantly different from titanium dioxide-instilled mice.
Figure 5. Detection of alveolar macrophage-associated ICAM-1. Mice were exposed to 2 mg/mouse (A) silica, (B) titanium dioxide, or (C) saline and sacrificed 5 days postinstillation. Brown areas indicate areas positive for ICAM-1 by staining with a monoclonal antibody specific for mouse ICAM-1. Photomicrographs are shown at × 400.
relationship between cell trafficking and ICAM-1 expression. Figure 7A demonstrates that silica exposure significantly increased the number of lavageable neutrophils (1, 3, 5, and 7 days after exposure) and macrophages (3, 5, and 7 days after exposure). With time, the increase in AM-associated ICAM-1 (Figure 4) and sICAM-1 (Figure 6) paralleled the increase in the number of AM. Figure 7B demonstrates that titanium dioxide exposure also significantly increased the number of lavageable neutrophils and macrophages, but well below that observed following silica exposure. There was no apparent association between the low level of sICAM-1 detected (Figure 6) and inflammatory cell influx in these mice.

**Discussion**

Levels of ICAM-1 were increased in lung tissue of mice following exposure to the toxic particle, silica, but not to the nuisance dust, titanium dioxide. This increase in ICAM-1 could be localized by immunohistochemistry to aggregates of pulmonary macrophages and to type II epithelial cells. Exposure to silica also elicited increased expression of TNF-α, which appeared to be localized to the same areas of the lung with increased ICAM-1 expression. Immunocytochemical analysis of cells isolated by BAL showed that ICAM-1 expression was increased in AM from animals exposed to silica. In addition, an increase in soluble ICAM-1 was measured in the cell-free BAL fluid following silica exposure. These increases in ICAM-1 were not detected to the same extent in mice exposed to the nuisance dust, titanium dioxide.

Detection of ICAM-1 expression associated with lavaged AM was greatest 5 days after silica exposure, whereas detection of increased ICAM-1 in lung homogenates (ELISA) and in lung tissue (immunohistochemistry) was greatest on day 1. Increased ICAM-1 expression associated with AM may reflect elevated ICAM-1 expression on newly recruited AM, as the peak in the number of lavaged AM was also seen on day 5 (Figure 7A). The peak in detection of sICAM in lung lavage fluid 5 days after silica (Figure 6) also appears to reflect the increased number and ICAM-1 expression on these cells at this time. Increased ICAM-1 expression in lung tissue, on the other hand, can be localized not only to AM, but also to interstitial macrophages and type II cells. The marked increase in ICAM-1 expression in lung parenchyma may reflect activation of resident cells by silica particles that are passing across the alveolar epithelial barriers prior to reaching afferent lymphatic channels (27). Past work in this mouse model documents detectable silica in the mediastinal lymph nodes as early as 1 day after silica exposure (28).

ICAM-1 is elevated in several models of antigen independent lung injury. The role of ICAM-1 in these models, as in other models of lung injury, is in participation in the inflammatory response generated by toxic insults. ICAM-1, through its interaction with its ligands, the β2-integrins (CD11a/CD18 or CD11b/CD18) on the surface of leukocytes, function in the adherence and migration of leukocytes (20). Studies have shown that an increase in ICAM-1 expression on the surface of endothelial cells is associated with an increase in neutrophil adhesion (19,29) and transendothelial migration (29,30). Exposure of mice to hyperoxia increased pulmonary ICAM-1 protein (14,31,32) and mRNA (32), whereas airway epithelial cells in mice increase ICAM-1 protein after 3 hr exposure to ozone (33). Intratracheal injection of Sephadex beads (Pharmacia, Uppsala, Sweden) in a murine foreign body granulomatous lung model increased expression of ICAM-1 in alveolar inflammatory cells and on lung parenchymal tissue (34) and exposure of rats to oleic acid also increased pulmonary ICAM-1 protein levels (25).

The role of increased expression of ICAM-1 associated with AM following silica exposure remains unclear. However, the presence of ICAM-1 on the surface of AM may indicate a level of silica-induced cell activation on newly recruited AM. Rothlein et al. (35) have shown that cross-linking of ICAM-1 on the surface of peripheral blood mononuclear leukocytes with a monoclonal anti-ICAM-1 antibody results in the generation of an oxidative burst, which suggests the capability of ICAM-1 to deliver a transmembrane signal. Others have also documented an association between increased AM ICAM-1 expression in fungi-activated AM (36), in
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AM from asthma patients (37), and AM from patients with active sarcoidosis (38).

Interestingly, we did not detect a marked increase in endothelial cell ICAM-1, yet previous studies in our lab demonstrated that passive immunization of mice with an antibody directed against ICAM-1 reduced silica-induced neutrophil influx into the airways by approximately 50% (24). The increased levels of ICAM-1 on the membrane of AM may promote interactions with other leukocytes (e.g., neutrophils), further stimulating these macrophages to perpetuate the inflammatory response. Passive immunization with anti-ICAM-1 antibody may have interfered with this process and thus reduced the phlogistic signals responsible for the migration of neutrophils.

Silica exposure also increased levels of sICAM-1 protein in the BAL fluid. sICAM-1 detected in the BAL fluid may be from pulmonary leukocytes (21) or alveolar epithelial cells (22). Because we detected ICAM-1 associated with AM following silica exposure, these cells are the most likely source in our model. This form of ICAM-1 is probably the result of proteolytic cleavage of cell-bound ICAM-1 close to the cell membrane, as it has been reported to contain most of the extracellular portion of the membrane-bound form (21). It may also be the result of an alternatively spliced form of mRNA lacking a transmembrane domain (39). The role for sICAM-1 remains unknown. Others have speculated that plasma sICAM-1 may bind to activated neutrophils (PMNs), thus reducing their adherence to endothelial cells and subsequent extravasation (21). Its increase in BAL fluid may be a marker of fibrosis (23), inflammation, tissue damage, and nonspecific proteolysis, rather than serving a physiological role (21). Indeed, the small increase in sICAM detected in BAL of titanium dioxide-exposed mice may reflect the limited inflammatory response observed with this low toxicity particle.

Cytokines TNF-α, IL-1, and IFN-γ upregulate ICAM-1 expression on alveolar epithelial and endothelial cells (15–17). The ability of these cytokines to promote the recruitment of PMNs may be due to their ability to upregulate adhesion molecule expression (40,41). These cytokines, which increase ICAM-1 expression, are also elicited from macrophages exposed in vitro or in vivo to silica. Driscoll et al. (2) have shown that TNF-α release is increased from AM lavaged from rats exposed to silica but not to titanium dioxide. More recently Ohtsuka et al. (3) reported TNF-α release from AM from silica-injected mice. Furthermore, Figuer et al. (42) have shown that passive immunization of mice with an antibody directed against TNF-α reduces silica-induced collagen deposition. Cytokines elicited by silica exposure may also be responsible for increased ICAM-1 expression on type II epithelial cells. Kang et al. (17) demonstrated in mice that nasal instillation of either TNF-α or IFN-γ increased ICAM-1 expression on type II epithelial cells by ~20-fold. This induction may be the result of differentiation of type II cells to replace injured type I epithelial cells (43) and/or a response to cytokines released during inflammation. It is well documented that exposure of type II cells to silica initiates repair, replication, and growth of these cells (44).

Thus, it can be speculated that the increase in ICAM-1 following silica exposure may be due in part to an increased expression of cytokines such as TNF-α. The apparent colocalization of both ICAM-1 and TNF-α by immunohistochemistry provides evidence to support this proposal. Increased TNF-α expression on pulmonary macrophages and on type II epithelial cells may represent the presence of TNF-α bound to its receptor, which in turn increases the expression of ICAM-1 at the same histopathological sites. The inability of titanium dioxide to increase ICAM-1 levels may pertain to its inability to substantially increase expression of TNF-α (2).

This study documents that exposure of mice to the toxic particle, silica, specifically increases ICAM-1 expression in the lungs, which can be localized to pulmonary macrophages and type II epithelial cells. Increased TNF-α expression appears to be colocalized to the same histopathological loci with increased ICAM-1 expression. Increased ICAM-1 expression also appears to be increased on newly recruited AM with subsequent shedding of cell bound ICAM-1 into the BAL fluid. Future studies will continue to focus on the mechanism of induction and the specificity/sensitivity of increased ICAM-1 expression following exposure to other toxic inorganic particles.

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