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α-Quartz-Induced Chemokine Expression by Rat Lung Epithelial Cells

Effects of in Vivo *and* in Vitro *Particle Exposure*

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Chemokines are chemotactic cytokines that can play a key role in leukocyte recruitment to sites of tissue injury or infection. Previous studies have demonstrated that exposure to α-quartz as well as other noxious particles increases chemokine gene expression in rat lung, although the cells responsible for chemokine expression and the mechanisms underlying this response have remained unclear. The present studies demonstrate that exposure of rats to α-quartz induced expression of mRNA for the chemokine macrophage-inflammatory protein (MIP)-2 in epithelial cells lining the terminal bronchioles and alveolar ducts as well as macrophages and alveolar type II cells in the more distal lung. Treatment of rats with an anti-MIP-2 antiserum before α-quartz exposure markedly attenuated neutrophilic infiltration of the lungs demonstrating an important role for MIP-2 in α-quartz-induced pulmonary inflammation. In vitro exposure of primary cultures of rat alveolar type II cells or the rat alveolar type II cell line RLE-6TN to tumor necrosis factor-α, endotoxin, or α-quartz increased mRNA for MIP-2 as well as the structurally and functionally similar chemokine cytokine-induced neutrophil chemoattractant but not the chemokine MIP-1α. The α-quartz-induced increase in epithelial MIP-2 mRNA resulted, at least in part, from increased gene transcription and was associated with the release of active MIP-2 protein. Induction of RLE-6TN MIP-2 and cytokine-induced neutrophil chemoattractant mRNA expression was not unique to α-quartz, being also increased by crocidolite asbestus fibers but not by titanium dioxide or MMVF-10 glass fibers. These findings indicate that epithelial cells contribute to chemokine expression in rat lung after exposure to α-quartz and potentially other noxious particles and suggest that α-quartz-activated MIP-2 expression in vivo results, at least in part, from a direct action of the particles on the lung epithelium. (Am J Pathol 1996, 149:1627–1637)

Exposure to respirable crystalline silica particles can result in an inflammatory and fibrotic lung disease, which, when manifested in its accelerated or acute forms, can be rapidly debilitating and fatal.[1] Studies using animal models of silicosis indicate that inflammatory cells and, in particular, neutrophils contribute to lung tissue injury resulting from crystalline silica exposure.[2] In this respect, it has been suggested that members of a supergene family of chemotactic cytokines known as chemokines contribute to the pulmonary recruitment of inflammatory cells after exposure to α-quartz, one of the polymorphic forms of crystalline silica.[3–5] Chemokines are small (ie, 8 to 10 kd) heparin-binding proteins that possess a conserved four-cysteine motif.[6–8] Based on structural, functional, and genomic considerations, chemokines can be divided into two subgroups designated as C-X-C or C-C chemokines.[6–8] The C-X-C chemokines, which include interleukin (IL)-8 and macrophage inflammatory protein (MIP)-2, typically exhibit neutrophil chemotactic activity[6–8] whereas the C-C chemokines, which include MIP-1α and monocyte chemotactic protein (MCP)-1, exhibit bioactivities that include chemotactic activity for

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monocytes and lymphocytes.[7,8] Recent studies on α-quartz-induced pulmonary inflammation have shown that expression of the chemokines MIP-2, cytokine-induced neutrophil chemoattractant (CINC), and MIP-1α increases dramatically in rat lungs shortly after α-quartz exposure.[3–5]

At present, the sources of chemokines in the lung after α-quartz exposure remain poorly defined as do the processes underlying α-quartz-induced activation of chemokine expression. There is evidence that one pathway for increased chemokine expression in the lung and other tissues involves activation of macrophages to release the cytokines tumor necrosis factor (TNF)-α and IL-1, which then act via autocrine and/or paracrine pathways to stimulate release of chemokines by cells including macrophages, epithelial cells, and fibroblasts.[4,5,9–11] An additional or alternate pathway by which α-quartz and other inhaled agents may influence lung chemokine expression is through a direct action on the lung epithelium. Like alveolar macrophages, epithelial cells are directly and continually exposed to the external environment, and these cells are known to respond to a diversity of stimuli *in vitro* with production of chemokines such as IL-8, MIP-2, CINC, and MCP-1.[3,9–11] Thus, it is possible that epithelial cells could respond to α-quartz particles with release of chemokines and, in this manner, facilitate inflammatory cell recruitment.

In the present studies, we used a rat model of silicosis to investigate the role of lung epithelial cells in α-quartz-induced lung chemokine expression. *In vivo* studies were conducted using *in situ* hybridization and passive immunization techniques to identify the lung cells that express the chemokine MIP-2 after α-quartz exposure and to assess the importance of MIP-2 in α-quartz-elicited neutrophil recruitment. *In vitro* studies were conducted to assess the role of direct particle/epithelial cell interactions in the *in vivo* response to α-quartz as well as to evaluate the extent to which the *in vitro* effects of α-quartz were particle specific. Our findings support an important role for chemokines in pulmonary inflammation and indicate that epithelial cells likely act as key effectors of the chemokine response. In addition, the *in vitro* results support a role for a direct effect of α-quartz, and potentially other particles, on activation of epithelial cell chemokine expression *in vivo.*

Materials and Methods

α-Quartz Intratracheal Instillation Studies

Specific pathogen-free male F344 rats (Charles River Breeding Laboratories, Kingston, NY), 12 to 14 weeks old and ~200 g body weight, were intratracheally instilled with saline or a saline suspension of 2 mg of α-quartz at a dose volume of 1 ml/kg body weight as described in detail elsewhere.[3] The α-quartz (Min-U-Sil, Pennsylvania Glass Sand Corp., Pittsburgh, PA) had a median diameter ± geometric standard deviation (GSD) of 0.9 ± 1.8 μm and a surface area of 4.5 m^2/g and was heated for 2 hours at 200°C for sterilization before use. To evaluate MIP-2 mRNA expression *in situ*, rats were killed by intraperitoneal injection of sodium pentobarbital (50 mg/kg) 2 or 24 hours after saline or α-quartz instillation, and the lungs were removed and infused via the trachea with 10% neutral buffered formalin to 25 cm pressure. In studies to examine the contribution of MIP-2 to α-quartz-induced lung inflammation, groups of three rats were pretreated intraperitoneally with 1 ml of saline, normal rabbit serum, or a rabbit anti-murine MIP-2 antiserum. At 2 hours after pretreatment, the rats were intratracheally instilled with saline or a saline suspension of 1 mg of α-quartz (dosing volume = 1 ml/kg), and 24 hours after dosing, the animals were killed by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Bronchoalveolar lavage (BAL) was performed, and differential cell counts were made on the BAL fluid cell population as described in detail elsewhere.[3]

In Situ *Hybridization*

Linearized plasmid DNAs of a homologous cDNA clone for rat MIP-2[8] was used as template for the preparation of cRNA probes. Sense and anti-sense orientations were confirmed on Northern blots. cRNA synthesis reactions were a modification of those described by DeLeon et al.[12] Final concentrations of the synthesis reactions were 40 mmol/L Tris-HCL (pH 7.5), 6 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 0.5 mmol/L unlabeled nucleoside triphosphate (NTP), 5 μmol/L [^{32}P]NTP, 20 μmol/L unlabeled NTP, 2 mmol/L spermidine, 100 μg/ml bovine serum albumin, 250 mg/ml template DNA, and 1500 U/ml RNA polymerase. Reactions were incubated at 38°C for 90 minutes, the DNA template was digested with RNAse-free DNAse I (Promega, Madison, WI), and then the probe was extracted with an equal volume of phenol/chloroform (1:1), precipitated out of ethanol, and resuspended in diethylpyrocarbonate-treated water. Full-length transcripts were approximately 1.4 to 1.5 kb; before hybridization, limited alkaline hydrolysis of RNA probes was performed to reduce transcript length to 0.1 to 0.3 kb.

Lung tissue sections (2 to 3 μm) were mounted on uncoated glass slides and deparaffinized in xylene

and rehydrated in graded ethanol. After treatment with 0.25% acetic anhydride, 0.1 mol/L glycine, and 2× standard saline citrate (SSC), slides were dehydrated through graded ethanol. Tissue sections were treated for 30 minutes at 37°C with 1 μg/ml proteinase K, washed, and dipped in fresh 0.25% acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0) for 10 minutes. After dehydration through a series of ethanol washes, the sections were dried and hybridized overnight at 56°C in 50% formamide, 0.3 mol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 1× Denhardts solution, 10% dextran sulfate, 0.5 mg/ml yeast tRNA, and 0.3 μg/ml cRNA probe. After hybridization, slides were washed twice in 1× SSC (sodium chloride, sodium citrate) for 10 minutes. Sections were treated with RNAse A (20 μg/ml) in RNAse buffer (0.5 mol/L NaCl, 10 mmol/L Tris-HCl, and 1 mmol/L EDTA, pH 7.5) for 30 minutes at 37°C. Slides were passed through 30-minute washes of 37°C RNAse buffer, 0.1× SSC at room temperature, 0.1× SSC at 68°C, and 0.1× SSC at room temperature. The slides were passed through a series of graded ethanol washes and dried. Autoradiography was performed; ^{32}P hybridizations were exposed for 14 days and then counterstained with hematoxylin and eosin.

Culture and in Vitro *Exposure of Rat Alveolar Epithelial Cells*

In vitro studies were conducted using the rat alveolar epithelial type II cell line RLE-6TN and, in some experiments, primary cultures of rat alveolar type II cells. The RLE-6TN cell line was derived as a spontaneous transformant from a culture of rat alveolar type II cells and has been described in detail.[13] RLE-6TN cells retain several characteristics of freshly isolated rat alveolar type II cells and are karyotypically stable, exhibiting a near diploid chromosome number.[13] The RLE-6TN cells were maintained in 25-cm^2 tissue culture flasks and grown in RLuE medium (Biological Research Facility and Faculty, Ijamsville, MD) which is a Ham's-F12-based medium supplemented with insulin, pituitary extract, transferrin, and fetal bovine serum (FBS; see Biological Research Facility and Faculty product catalog for details of medium composition). Rat alveolar type II cells were isolated and cultured as described previously.[14] Briefly, the heart, lungs, and trachea were removed from male Fischer 344 rats (Charles River Laboratories), and the lungs were perfused via the pulmonary artery with a sterile buffered salt solution (BSS; 125 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L $NaHPO_4$, 17 mmol/L Hepes, 0.1% glucose, pH 7.4). The lungs were lavaged five times with 10 ml of BSS and twice with 10 ml of BSS containing 2.5 mmol/L $CaCl_2$ and 1.2 mmol/L $MgSO_4$ (Ca-Mg BSS). A Pronase (Sigma Chemical Co., St. Louis, MO) solution (0.25% in Ca-Mg BSS) was instilled into the trachea for a total of 15 minutes, the lungs minced into ~1- to 4-mm pieces, and the minced tissue was filtered through sterile nylon filters. The resultant cell suspensions were centrifuged at 500 × *g* for 10 minutes, and the cell pellet was resuspended in Ham's F12 medium (Gibco, Gaithersburg, MD) containing 2% FBS and layered over a Nycodenz (Accurate Chemical, Westbury, NY) gradient and centrifuged for 20 minutes at 1500 × *g* (15°C). The epithelial-cell-containing layer was removed and resuspended in RLuE medium. Alveolar type II cell numbers were characterized by alkaline phosphatase and/or modified Papanicolaou stains. Freshly isolated type II cells were seeded at 1 × 10^5 epithelial cells/35-mm well in RLuE medium. After 3 days in culture, >97% of the cells stained for alkaline phosphatase, with the remaining cells having the morphological appearance of macrophages.

Initial experiments were conducted to compare the expression of a panel of neutrophil chemotactic cytokines in RLE-6TN cells and primary culture rat alveolar type II cells in response to the agonists TNF-α and endotoxin. These experiments were performed to further support the use of RLE-6TN cells for evaluating the potential effects of α-quartz on rat alveolar type II cell chemokine expression *in vivo.* Briefly, confluent cultures of the RLE-6TN cells or 3-day-old primary cultures of rat alveolar type II cells were placed in Ham's F12 medium containing 2% FBS overnight followed by exposure to 50 ng/ml recombinant murine TNF-α (Genzyme, Cambridge, MA) or 50 ng/ml lipopolysaccharide (Sigma) for 6 hours, after which RNA was extracted from the cells as described by Chomczynski and Sacchi[15] for reverse transcriptase polymerase chain reaction (RT-PCR) analysis.

To characterize the effect of *in vitro* α-quartz exposure on rat epithelial cell chemokine expression, RLE-6TN cells were grown to confluence in RLuE medium and then placed in Ham's F12 medium containing 2% FBS overnight followed by exposure to suspensions of mineral dust particles at doses of 2, 6, 20, and 60 μg of α-quartz/cm^2 of the culture dish for a period of 6 hours. The α-quartz was the same as that described above for the *in vivo* exposure studies and was heated to 200°C for 2 hours for sterilization and then suspended in Ham's F12 medium containing 2% FBS. After 6 hours of exposure, epithelial-

cell-conditioned medium was collected for analysis of lactate dehydrogenase (LDH) activity as an indicator of cytotoxicity, and RNA was extracted from the epithelial cells for analysis of chemokine gene expression. In some experiments, conditioned medium was evaluated for neutrophil chemotactic activity and the presence of MIP-2 protein.

To determine whether the effects of α-quartz on RLE-6TN cell chemokine expression were particle-type-specific, these cells were exposed for 6 hours as described above to titanium dioxide (Anatase, Fisher Scientific, Fairlawn, NJ) having a median diameter ± GSD of 0.18 ± 1.6 μm and surface area of 8.8 m^2/g; MMVF-10 glass fibers (Fiber Repository of the Thermal Insulation Manufacturers Association, Standford, Canada) having a median diameter ± GSD of 0.11 ± 1.8 μm and length, 65% < 10 μm; and crocidolite asbestos (gift from Dr. Morton Lippmann, New York University) with a median diameter ± GSD of 0.28 ± 1.8 μm length, 23% < 10 μm, and surface area of 3.2 m^2/g. After 6 hours of exposure, epithelial-cell-conditioned medium was collected for analysis of LDH activity, and RNA was extracted from the epithelial cells for analysis of chemokine gene expression. All test particles were heated to 200°C for 2 hours for sterilization and then suspended in Ham's F12 medium containing 2% FBS.

RT-PCR Analysis of Chemokine Expression

MIP-1α, MIP-2, and CINC mRNA transcript levels were assessed by PCR amplification of the MIP-1α, MIP-2, and CINC cDNAs as described in detail elsewhere.[3] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was evaluated as an internal control. RNA from the lungs of an untreated F344 rat and from an F344 rat instilled with 10 μg of lipopolysaccharide and sacrificed 6 hours after exposure were analyzed concurrent with unknown RNA samples as negative and positive controls for chemokine mRNA expression. The primers, designed from the published sequences for MIP-1α,[16] MIP-2,[8] CINC,[17] and GAPDH,[18] were as follows: MIP-1α, 5′-GATGTATTCTTGGACCCAGGT-3′ and 5′-TATGGAGCTGACACCCCGAC-3′; MIP-2, 5′-GGCACATCAGGTACGATCCAG-3′ and 5′-ACCCTGCCAAGGGTTGACTTC-3′; CINC, 5′-TTCTCTGTGCAGCGCTGCTG-3′ and 5′-CAGGGTCAAGGCAAGCCTCG-3′; GAPDH, 5′-CAGGATGCATTGCTGACAATC-3′ and 5′-GGTCGGTGTGAACGGATTTG-3′.

Nuclear Run-On Analysis

MIP-2 gene transcription in RLE-6TN cells was analyzed by nuclear run-on analysis as described previously.[19] Briefly, confluent cultures of RLE-6TN cells were exposed to 20 μg/cm^2 α-quartz for 1 or 4 hours, nuclei were prepared by Nonidet P-40 lysis of treated cells, and the nuclear extracts were frozen at −80°C for use in nuclear run-on assays. The nuclear run-on assay was performed by incubation of prepared nuclear extracts with [^{32}P]UTP (Amersham, Arlington Heights, IL) to label nascent transcripts. Transcripts from the genes of interest (ie, MIP-2, c-*myc*, and GAPDH) were identified and quantitated by hybridization of the labeled RNA to nitrocellulose filters containing immobilized plasmid DNA, rat MIP-2, c-*myc*, and GAPDH cDNA followed by visualization of the hybridized RNA by autoradiography. GAPDH and c-*myc* gene transcription was examined along with MIP-2 as the former represents a constitutively transcribed gene and the latter a gene whose transcription is activated by a variety of stimuli.

Neutrophil Chemotaxis

Conditioned medium from α-quartz-exposed RLE-6TN cells was analyzed for neutrophil chemotactic activity as described previously.[20] Briefly, neutrophils were isolated from peripheral blood obtained from the abdominal aorta of F344 rats and suspended in Gey's BSS (Gibco/BRL, Gaithersburg, MD). Chemotaxis assays were conducted using a 48-well microchemotaxis assembly (Neuroprobe, Cabin John, MD) with the lower well filled with 25 μl of RLE-6TN-cell-conditioned medium, Gey's BSS (negative control), or 1% zymosan-activated rat serum, the latter a positive chemotactic stimulus. The upper wells were filled with 50 μl of cell suspensions containing 1 × 10^5 neutrophils. The upper and lower wells were separated by polycarbonate filters (5-μm pores; Neuroprobe). The chemotaxis chambers were incubated for 30 minutes at 37°C, 5% CO_2, after which the filters were removed, fixed in methanol, and stained with Diff Quik (Sigma). Filters were scored for the number of cells having migrated through the filter in five ×400 microscope fields. In some experiments, epithelial-cell-conditioned media were pretreated with either a 1/100 dilution (in phosphate-buffered saline; PBS) of rabbit anti-murine MIP-2 antiserum or normal rabbit serum before evaluating for chemotaxic activity.

Immunoblotting for MIP-2

RLE-6TN-cell-conditioned medium (500 μl) was blotted onto nitrocellulose using a slot blot apparatus (Gibco/BRL). The blot was incubated for 60 minutes at room temperature in a solution containing 5% nonfat milk, 50 mmol/L Tris-HCl, 0.5 mol/L NaCl, pH 7.5, followed by incubation in the same solution containing a 1:250 dilution of rabbit anti-murine MIP-2 antiserum for 60 minutes. This anti-MIP-2 antiserum has been shown previously to react specifically with recombinant rat MIP-2.[21] The blot was washed three times in TN buffer (50 mmol/L Tris-HCl, 0.5 mol/L NaCl, pH 7.5) and incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) diluted 1:50 with TN buffer containing 0.3% bovine serum albumin. The blot was washed three times in TN buffer containing 0.1% Triton X-100 (Sigma) and incubated in streptavidin-horseradish peroxidase (1:250 in PBS) for 60 minutes followed by addition of 3,3′-diaminobenzidine (Vector), and the color reaction was allowed to develop for 10 minutes.

Statistical Analysis

When appropriate, data were analyzed by analysis of variance with group differences determined using the Newman-Keuls test.[22]

Results

In Vivo *α-Quartz Exposure and MIP-2 Chemokine Expression*

Intratracheal instillation of rodents with α-quartz produces a chronic/active inflammation that progresses to a granulomatous and fibrotic lesion resembling accelerated or acute silicosis.[23–25] We used intratracheal instillation of α-quartz to characterize the rat lung cells expressing the chemokine MIP-2 after acute exposure to this mineral dust. *In situ* hybridization analysis of rat lung tissue 2 and 24 hours after intratracheal instillation of the saline vehicle revealed no remarkable expression of MIP-2 mRNA (Figure 1A). In the lungs of α-quartz-exposed rats, marked expression of MIP-2 mRNA was detected in epithelial cells in the terminal bronchioles and alveolar ducts at both 2 and 24 hours after α-quartz exposure (Figure 1, B, C, and E). Epithelial MIP-2 gene expression appeared slightly less at 24 compared with 2 hours after α-quartz exposure. Also exhibiting MIP-2 gene expression were cells in more distal alveoli, some of which appeared by their morphology and location to be alveolar type II epithelial cells and others alveolar macrophages. The specificity of the *in situ* analysis for MIP-2 mRNA was demonstrated by the absence of hybridization in tissue probed with cRNA in the sense orientation (Figure 1D).

To investigate the contribution of MIP-2 to α-quartz-induced pulmonary inflammation, rats were treated with an anti-MIP-2 antisera or nonimmune sera before α-quartz treatment. Figure 2 presents the numbers of neutrophils quantitated in BAL fluid of rats 24 hours after intratracheal instillation of α-quartz or saline. As reported previously,[3] marked increases in neutrophils were observed after α-quartz but not saline exposure. Pretreatment of rats with antibody to MIP-2 significantly attenuated the neutrophilic inflammatory response, an effect not observed in rats pretreated with normal rabbit serum.

Chemokine Expression by Rat Alveolar Epithelial Cells in Vitro

Experiments were conducted comparing chemokine expression in the rat alveolar type II cell line, RLE-6TN cells, and primary cultures of rat alveolar type II cells. mRNA for the chemokines MIP-1α, MIP-2, or CINC was not detected in unstimulated cultures of primary rat alveolar type II cells or RLE-6TN cells (Figure 3, A and B). In both primary alveolar type II and RLE-6TN cultures, exposure to 50 ng/ml TNF-α or lipopolysaccharide increased steady-state levels of mRNA for MIP-2 and CINC but not MIP-1α. The absence of detectable MIP-1α expression in primary alveolar type II cell cultures indicates that any macrophage contamination of these cultures did not remarkably contribute to the RT-PCR analysis for chemokine expression as macrophages express high levels of MIP-1α mRNA after stimulation with 50 ng/ml TNF-α.[3] The similar patterns of MIP-1α, MIP-2, or CINC (and MCP-1, data not shown) expression in RLE-6TN cells and primary cultures of rat alveolar type II epithelial cells under resting and stimulated conditions supports the use of the former to investigate potential effects of particles on chemokine expression by rat alveolar type II cells.

Effect of in Vitro *α-Quartz Exposure on RLE-6TN Chemokine Expression*

To investigate the possibility that induction of MIP-2 gene expression in rat lung epithelial cells after *in vivo* α-quartz exposure might result from a direct interaction between the particles and epithelial cells, *in vitro* exposure studies were conducted using RLE-

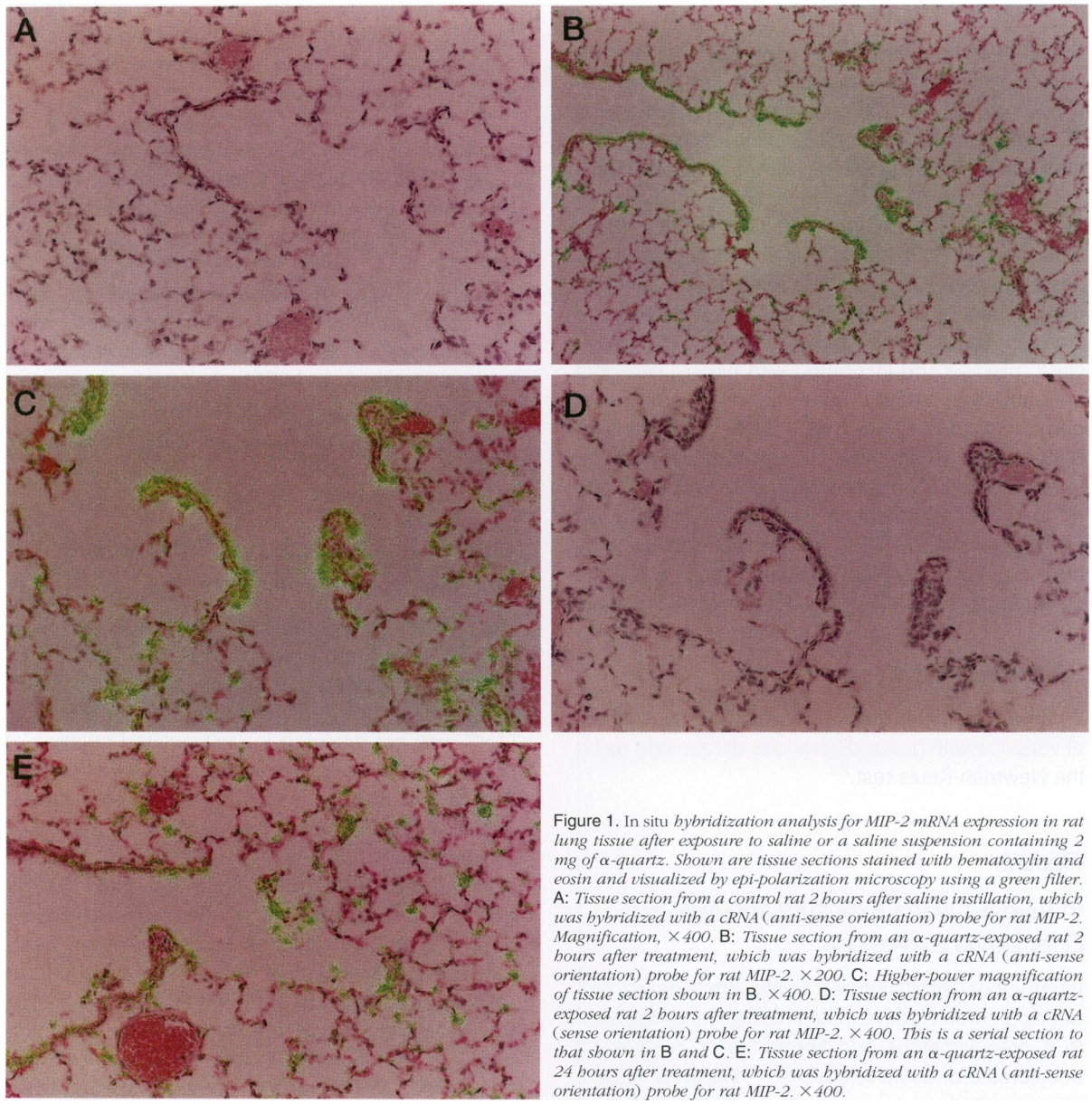

Figure 1. In situ *hybridization analysis for MIP-2 mRNA expression in rat lung tissue after exposure to saline or a saline suspension containing 2 mg of α-quartz. Shown are tissue sections stained with hematoxylin and eosin and visualized by epi-polarization microscopy using a green filter.* A: *Tissue section from a control rat 2 hours after saline instillation, which was hybridized with a cRNA (anti-sense orientation) probe for rat MIP-2. Magnification, ×400.* B: *Tissue section from an α-quartz-exposed rat 2 hours after treatment, which was hybridized with a cRNA (anti-sense orientation) probe for rat MIP-2. ×200.* C: *Higher-power magnification of tissue section shown in* B. *×400.* D: *Tissue section from an α-quartz-exposed rat 2 hours after treatment, which was hybridized with a cRNA (sense orientation) probe for rat MIP-2. ×400. This is a serial section to that shown in* B *and* C. E: *Tissue section from an α-quartz-exposed rat 24 hours after treatment, which was hybridized with a cRNA (anti-sense orientation) probe for rat MIP-2. ×400.*

6TN cells and, in some experiments, primary cultures of rat alveolar type II cells. Exposure of RLE-6TN cells to 60 $\mu g/cm^2$ α-quartz resulted in significant LDH release (Figure 4). This LDH response was 57% of the total LDH release determined after lysis of RLE-6TN cells with Triton X-100. Treatment of RLE-6TN cells with ≤20 $\mu g/cm^2$ α-quartz did not significantly increase LDH release (Figure 4) but did increase steady-state levels of MIP-2 and CINC mRNA (Figure 5A). Similar to observations with TNF-α or endotoxin, α-quartz did not increase mRNA for MIP-1α. When primary cultures of rat alveolar type II cells were exposed to 20 $\mu g/cm^2$ α-quartz, consistent with responses of RLE-6TN cells, increases in MIP-2 and CINC but not MIP-1α mRNA were detected (Figure 5B). Nuclear run-on analysis demonstrated that exposure to 20 $\mu g/cm^2$ α-quartz increased transcription of the MIP-2 gene as well as the proto-oncogene c-*myc* in RLE-6TN cells (Figure 6). Increased gene transcription was detected 1 hour after exposure and remained elevated after 4 hours of exposure. Transcription of the GAPDH gene was constitutive and was not altered by α-quartz.

As shown in Figure 7, increased levels of immunoreactive MIP-2 protein and neutrophil chemotactic activity were detected in RLE-6TN-cell-conditioned medium after exposure to 20 or 60 $\mu g/cm^2$ α-quartz. The neutrophil chemotactic activity in the condi-

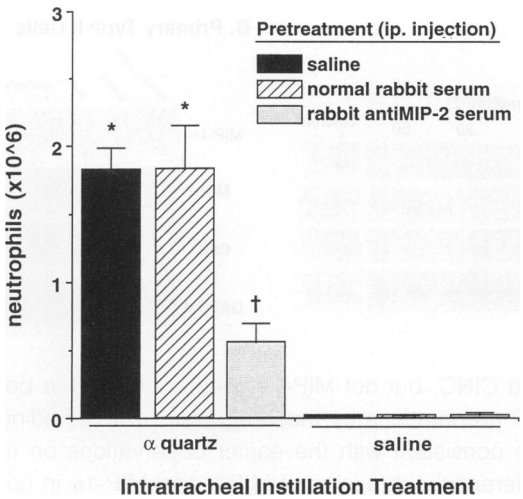

Figure 2. *Effect of passive immunization of rats with anti-MIP-2 antiserum on α-quartz-elicited neutrophilic pulmonary inflammation. Rats were pretreated intraperitoneally with saline, normal rabbit serum, or a rabbit anti-murine MIP-2 antiserum and subsequently intratracheally instilled with saline or a saline suspension of 1 mg of α-quartz. Animals were sacrificed 24 hours after instillation and subjected to bronchoalveolar lavage (BAL), and the number of BAL fluid neutrophils was determined. Results represent the mean ± SD; n = 3 rats/treatment. *Statistically significant difference from the respective saline control group mean; P < 0.05. †Statistically significant differences from the respective saline control group mean and the other α-quartz-treated groups; P < 0.05.*

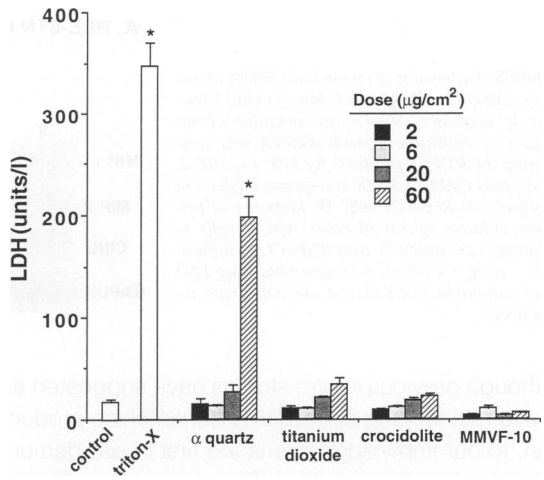

Figure 4. *Release of LDH by RLE-6TN cells exposed* in vitro *to particles. Cells were exposed for 6 hours to doses of 2 to 60 μg/cm² particles, and levels of LDH were determined in the culture media. Lysis of cells with Triton X-100 followed by analysis of LDH was used to determine total cellular LDH. Shown are the mean ± SD of triplicate cultures. *Statistically significant difference from the untreated control group.*

tioned medium from α-quartz-treated cells was reduced approximately 50% by treatment with a rabbit anti-murine MIP-2 antiserum but not by treatment with normal rabbit serum (Figure 7B).

Effect of Titanium Dioxide, Crocidolite, and MMVF-10 on Chemokine Expression

A 6-hour exposure to titanium dioxide or MMVF-10 glass fibers at levels of 2 to 60 μg/cm² had no detectable effect on RLE-6TN MIP-2 (Figure 8), nor did they increase LDH release (Figure 4). Crocidolite fibers at doses of 20 and 60 μg/cm² increased MIP-2 (Figure 8) and CINC (data not shown) mRNA whereas none of the exposures resulted in a significant increase in LDH (Figure 4).

Discussion

The pulmonary recruitment and activation of inflammatory cells is thought to play a key role in the pathogenesis of silicosis, a chronic interstitial lung disease resulting from exposure to various crystalline silica polymorphs.[1] Recently, we reported that α-quartz exposure increases MIP-2, CINC, and MIP-1α gene expression in rat lungs, implicating these chemokines as mediators of α-quartz-induced inflammation.[3–5] The present studies confirm and extend these earlier observations by identifying the cell types expressing MIP-2 mRNA in rat lungs after α-quartz exposure. MIP-2 mRNA expression was most striking in the terminal bronchiole and alveolar duct epithelium and was also apparent in the more distal lung in alveolar type II cells and macrophages. The localization of MIP-2 mRNA expression in the terminal bronchiole/alveolar duct region corresponds to a major site of particle deposition after inhalation or intratracheal instillation of particles.[26]

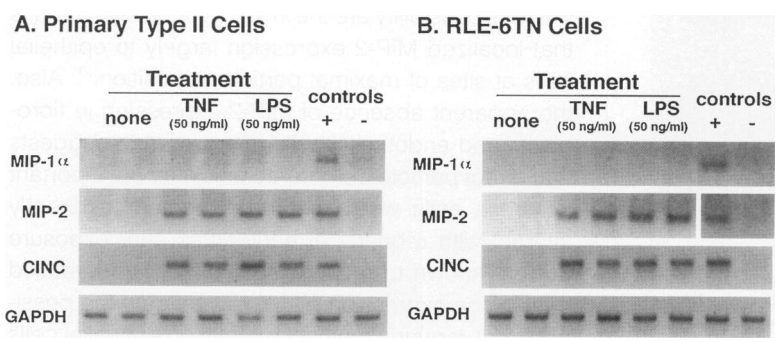

Figure 3. *Expression of chemokine mRNA by rat lung epithelial cells after exposure to 50 ng/ml TNF-α or 50 ng/ml endotoxin. Shown are negative photographs of ethidium-bromide-stained gels containing the RT-PCR products for MIP-1α, MIP-2, CINC, and GAPDH.* **A:** *Response of primary cultures of rat alveolar type II cells.* **B:** *Response of the rat alveolar type II epithelial cell line RLE-6TN. The + and − controls represent total lung RNA from endotoxin-instilled and naive rats, respectively.*

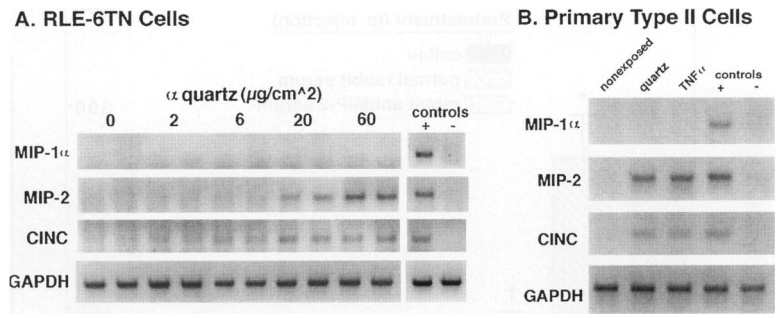

Figure 5. *Expression of chemokine mRNA by rat lung epithelial cells after a 6-hour* in vitro *exposure to α-quartz. Shown are negative photographs of ethidium-bromide-stained gels containing the RT-PCR products for MIP-1α, MIP-2, CINC, and GAPDH.* A: *Dose-responsive effects of α-quartz on RLE-6TN cells.* B: *Response of primary cultures of rat alveolar type II cells to α-quartz (20 μg/cm²) and TNF-α (50 ng/ml). The + and − controls represent total lung RNA from endotoxin-instilled and untreated rats, respectively.*

Although previous *in vitro* studies have suggested an *in vivo* role for lung epithelial cell chemokine production, to our knowledge this is the first *in situ* demonstration of increased chemokine gene expression in epithelial cells after exposure to an inflammatory agent. Having observed marked increases in lung MIP-2 mRNA expression after α-quartz, we conducted additional *in vivo* studies to examine the degree to which MIP-2 contributes to α-quartz-induced inflammation. The finding that pretreatment of rats with an anti-MIP-2 antiserum but not nonimmune serum reduces by approximately 60% the neutrophilic response to intratracheally instilled α-quartz provides more direct evidence that MIP-2 is a key mediator of α-quartz-induced pulmonary inflammation.

In response to the pro-inflammatory cytokines TNF-α or IL-1, mRNA for rat MIP-2 and the structurally and functionally related chemokine CINC is increased in a variety of cell types including macrophages, lung fibroblasts, and intestinal epithelial cells.[3,27,28] In contrast, expression of rat MIP-1α, a chemokine that is chemotactic for both macrophages and neutrophils, has been observed primarily in leukocytes.[3,6] In the present studies, we characterized the effects of TNF-α and bacterial endotoxin on the rat alveolar type II cell line RLE-6TN, and primary cultures of rat alveolar type II cells and found that steady-state levels of mRNA for MIP-2

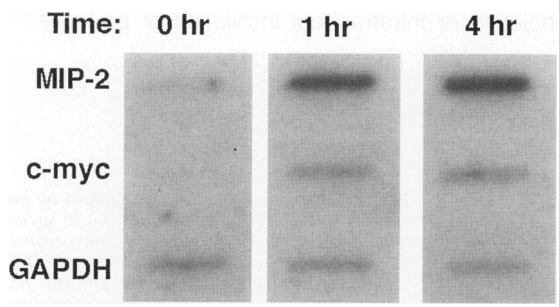

Figure 6. *Transcriptional analysis of MIP-2,* c-myc, *and GAPDH genes in RLE-6TN cells exposed* in vitro *to 20 μg/cm² α-quartz for 0, 1, or 4 hours. ³²P-labeled RNA transcripts were hybridized with cDNA for MIP-2,* c-myc, *and GAPDH immobilized on nitrocellulose, and the radioactivity was visualized by autoradiography.*

and CINC, but not MIP-1α, were increased in both cell preparations by these agonists. These findings are consistent with the earlier observations on the differential expression of MIP-2 and MIP-1α in nonleukocytic cells and extend previous findings that production of the chemokines IL-8 and MCP-1 is stimulated by TNF-α in the human lung carcinoma cell line A549.[10,11,29] The observation that endotoxin is also an agonist for epithelial cell MIP-2 and CINC expression suggests that the lung epithelium may be a key effector of inflammation via chemokine production in endotoxic sepsis and Gram-negative pneumonia, conditions characterized by massive tissue infiltration of neutrophils.

The precise mechanisms by which α-quartz exposure increases MIP-2 gene expression in rat lung epithelial cells *in vivo* is uncertain. This response may occur secondary to the release of other cytokines, the expression of which is increased by α-quartz. In this respect, we have reported that *in vitro* or *in vivo* α-quartz exposure increases production of TNF-α by rat alveolar macrophages.[4,25,30] In the present study, rat lung epithelial cells were shown to respond to TNF-α *in vitro* with increased expression of MIP-2 and CINC. Collectively, these findings suggest α-quartz-induced increases in MIP-2 *in vivo* may result, at least partly, via a cytokine network involving macrophage-derived TNF-α. An additional or alternative pathway for α-quartz-induced epithelial cell chemokine expression is a direct effect of the particles on epithelial release of chemokines. Consistent with this possibility are the *in situ* hybridization results that localized MIP-2 expression largely to epithelial cells at sites of maximal particle deposition.[26] Also, the apparent absence of MIP-2 expression in fibroblasts and endothelial cells after α-quartz suggests that direct particle-cell interactions may be important as these cells would not be expected to directly interact with α-quartz at early times after exposure but are known to respond to TNF-α with increased chemokine expression.[4,6,7] To investigate the possibility that α-quartz may act directly on epithelial cells

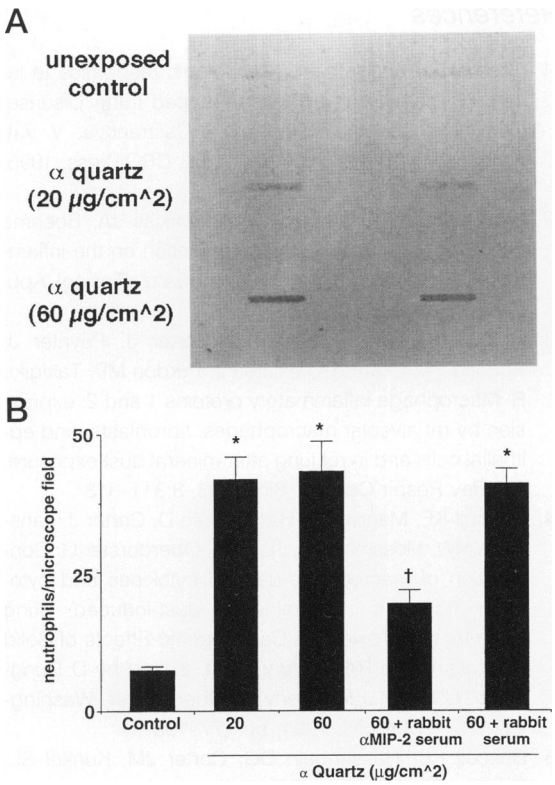

Figure 7. *Release of immunoreactive and bioactive MIP-2 protein by RLE-6TN cells after a 6-hour* in vitro *exposure to α-quartz.* A: *Immunoblot demonstrating increased MIP-2 protein in conditioned media from RLE-6TN cells exposed to 20 or 60 μg/cm² α-quartz; results are shown for samples from two RLE-6TN cultures.* B: *Neutrophil chemotactic response to medium from control (unexposed) RLE-6TN cells, medium from cells exposed to 20 or 60 μg/cm² α quartz, and medium from cells exposed to 60 μg/cm² α-quartz that was pretreated with a 1:100 dilution of rabbit anti-murine MIP-2 serum (αMIP-2) or normal rabbit serum. Shown are the mean ± SD of triplicate determinations on samples from three epithelial cultures/treatment. *Statistically significant difference from the control group. †Statistically significant differences from the control group and respective α-quartz-treated group.*

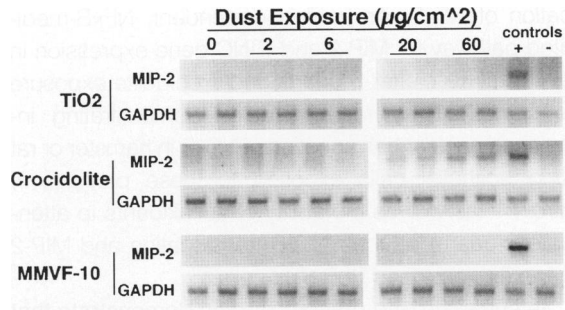

Figure 8. *Expression of chemokine mRNA by rat lung epithelial cells after a 6-hour* in vitro *exposure to titanium dioxide, crocidolite asbestos fibers, or MMVF-10 glass fibers. Shown are negative photographs of ethidium-bromide-stained gels containing the RT-PCR products for MIP-2 and GAPDH. The + and − controls represent total lung RNA from endotoxin-instilled and untreated rats, respectively.*

to activate chemokine expression, we conducted a series of *in vitro* studies using RLE-6TN cells and primary cultures of rat alveolar type II cells. The results demonstrate that α-quartz exposure increases expression of mRNA for MIP-2 and CINC by rat alveolar epithelial cells. The α-quartz-induced increase in epithelial MIP-2 mRNA levels resulted, at least in part, from increased gene transcription and was associated with release of immunoreactive and bioactive MIP-2 protein. These data clearly suggest that the induction of MIP-2 gene expression in rat lung epithelial cells after *in vivo* α-quartz exposure occurs, at least in part, as a result of a direct effect of α-quartz on these cells.

We found that *in vitro* activation of epithelial cell chemokine expression was not unique to α-quartz but was also not a property of all particles we examined. *In vitro* exposure of RLE-6TN to the less inflammatory dusts titanium dioxide or MMVF-10 glass fibers did not increase epithelial cell MIP-2 and CINC mRNA expression. In contrast, crocidolite asbestos fibers increased expression of these chemokines at exposure levels similar to those effective for α-quartz. This finding for crocidolite is consistent with those for the human lung carcinoma cell line A549, which was reported to respond to chrysotile and crocidolite fibers *in vitro* with production of the chemokine IL-8.[29] Importantly, like α-quartz, crocidolite fibers are highly inflammatory *in vivo*,[31,32] whereas titanium dioxide particles and MMVF-10 glass fibers are markedly less so.[3,33] Thus, using this limited panel of materials, rat lung epithelial cells were shown to respond differently to particles of high and low *in vivo* inflammatory activity with respect to chemokine production, suggesting that epithelial cell chemokine release may be a factor contributing to the greater *in vivo* inflammatory activity of some materials (eg, α-quartz and crocidolite).

The differential effect of α-quartz, crocidolite, titanium dioxide, and MMVF-10 glass fibers on MIP-2 and CINC expression provides insights into potential mechanisms underlying the epithelial chemokine response. Both α-quartz and crocidolite can give rise to reactive oxygen species, either as a result of their surface chemistry, particle-associated iron, and/or by stimulating the cellular generation of oxidants.[34,35] In this respect, the promoter region for MIP-2, CINC, and the structurally and functionally related human *gro* genes contains a binding element for the oxidant stress-responsive transcription factor NFκB.[36–38] The NFκB binding site has been shown to be both necessary and sufficient for MIP-2 and *gro* gene transcription in response to several stimuli including lipopolysaccharide, TNF-α, and/or IL-1.[36–38] Thus, it is likely that α-quartz- and crocidolite-induced transcription of MIP-2 and CINC is dependent, at least in part, on their ability to induce oxidative stress and stimulate nuclear translo-

cation of NFκB. An oxidant-dependent, NFκB-mediated pathway for MIP-2 and CINC gene expression in epithelial cells after α-quartz and crocidolite exposure is supported by recent studies demonstrating increased nuclear translocation of NFκB in hamster or rat lung epithelial cells exposed to these particles *in vitro*[39,40] as well as the ability of antioxidants to attenuate α-quartz-induced NFκB translocation and MIP-2 gene expression in RLE-6TN cells.[40]

In summary, the present studies demonstrate that exposure to α-quartz, a form of crystalline silica, induces expression of mRNA for the chemokine MIP-2 in rat lungs. MIP-2 gene expression was most striking in epithelial cells associated with the terminal bronchioles and alveolar ducts and was also apparent in macrophages and alveolar type II epithelial cells in the distal lung. The demonstration that pretreatment of rats with anti-MIP-2 antiserum reduced by approximately 60% the α-quartz-induced neutrophilic inflammatory response supports a key role for MIP-2 in α-quartz-induced inflammation. Although multiple mechanisms may contribute to epithelial cell MIP-2 gene expression after α-quartz exposure, α-quartz *in vitro* was shown to increase steady-state levels of mRNA for MIP-2 and the related chemokine CINC in both the rat alveolar type II cell line RLE-6TN and primary cultures of rat alveolar type II cells. α-Quartz-induced increases in epithelial MIP-2 mRNA expression resulted, at least in part, from increased gene transcription and was associated with release of bioactive MIP-2 protein. These results suggest that a direct effect of α-quartz may be important in activating rat epithelial cell MIP-2 gene expression *in vivo.* The *in vitro* activation of chemokine expression by epithelial cells was not unique to α-quartz, being also observed for crocidolite asbestos fibers but not for the relatively innocuous titanium dioxide particles or MMVF-10 glass fibers. Overall, these *in vivo* and *in vitro* observations indicate that rat lung epithelial cells are key contributors to chemokine expression in the lung after exposure to α-quartz and potentially other noxious particles. For some materials (ie, α-quartz and crocidolite), activation of lung epithelial cell chemokine expression may result, at least in part, from a direct effect of particles on the epithelium.

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