Cytokines and Particle-induced Inflammatory Cell Recruitment

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The inflammatory response is a key component of host defense. However, excessive or persistent inflammation can contribute to the pathogenesis of disease. Inflammation is regulated, in part, by cytokines, which are small, typically glycosylated proteins that interact with membrane receptors to regulate cellular processes such as proliferation, differentiation, and secretion. During the past 10 years studies in humans and experimental animals have demonstrated that a cytokine called tumor necrosis factor alpha (TNF-α) plays a key role in the initiation of inflammatory responses in the lung and other tissues, including inflammation resulting from inhalation of noxious particles. There is now compelling evidence that one of the pathways by which inhaled particles stimulate the recruitment and subsequent activation of inflammatory cells is through the activation of lung macrophages to release TNF-α. TNF-α then acts via paracrine and autocrine pathways to stimulate cells to release other cytokines known as chemokines, which are directly chemotactic to leukocytes and other cells that participate in inflammatory and wound healing responses. In addition to a TNF-α-mediated pathway, there is growing evidence that some particles such as quartz and crocidolite can directly activate lung epithelial cells to release chemokines such as macrophage inflammatory protein-2, cytokine-induced neutrophil chemoattractant, and interleukin-8. A direct stimulatory effect of particles on lung epithelium may represent an additional or alternate pathway by which inhaled particles may elicit inflammation in the lung. Recent studies have suggested that oxidative stress may be a component of the mechanism by which particles activate cytokine production in cells such as macrophages and epithelial cells. The contribution of oxidative stress to particle-induced cytokine gene expression appears to be mediated, at least in part, through activation of the transcription factor nuclear factor kappa B. — *Environ Health Perspect* 105(Suppl 5):1159–1164 (1997)

Key words: cytokines, tumor necrosis factor, chemokines, macrophage inflammatory protein, CINC, quartz, asbestos,nuclear factor kappa B

Introduction

Inflammation is a normally protective response to destroy, dilute, or isolate an eliciting agent and promote the repair of injured tissue. However, when inflammation is excessive or persistent it may cause tissue injury or organ dysfunction and may contribute to the pathogenesis of disease. A key component of inflammatory reactions is the accumulation and subsequent activation of leukocytes, a response that depends on an elaborate signaling network (*1*). Cytokines are one class of signaling molecules that regulate inflammatory processes and are important host factors influencing the response to noxious agents. Over the past 10 years considerable progress has been made in understanding the cytokines that contribute to the recruitment of inflammatory cells to the lung after exposure to noxious particles. In the following pages we summarize some of the evidence indicating a key role for the cytokine tumor necrosis factor alpha (TNF-α) as well as members of the chemokine cytokine family in particle-induced inflammation in the lung. The role of epithelial cells as primary effectors of cytokine production in the lung in response to particles, and the potential contribution of particle-induced oxidative stress to cytokine gene expression, are also discussed.

Tumor Necrosis Factor

In humans, TNF-α is synthesized as a 233 amino acid, approximately 26 kd precursor protein that is processed to a mature 157 amino acid, 17 kd protein (*2,3*). TNF-α can be produced by a variety of cells including macrophages, monocytes, polymorphonuclear leukocytes, smooth muscle cells, and mast cells (*4–6*). Alveolar macrophages are especially potent sources of TNF-α (*7*). TNF-α exerts its effects by interacting with two distinct cell membrane associated receptors with a trimeric TNF-α expressing the greatest bioactivity (*8,9*). TNF-α exhibits a variety of bioactivities, including inducing expression of the adhesion molecules such as E-selectin, ICAM-1 and VCAM-1; stimulating expression of cytokines including interleukin (IL)-1, IL-6, platelet-derived growth factor, and transforming growth factor beta; stimulating arachidonic acid metabolism; and activating leukocytes to release reactive oxygen and nitrogen species (*10–16*).

A role for TNF-α as a mediator of the respiratory tract's response to particles was suggested several years ago by *in vitro* studies that characterized the production of TNF-α by alveolar macrophages (Table 1). These studies demonstrated that some particles can activate alveolar macrophages to release TNF-α and indicated that the ability to activate macrophage TNF-α production *in vitro* corresponds with *in vivo* inflammatory activity. For example,

Table 1. Alveolar macrophage TNF-α production after *in vitro* exposure to various particulate materials.

Material	Source of macrophages	TNF-α response[a]	Reference
Endotoxin	Rat	+++++	(*17,18*)
Quartz	Rat	+++	(*17,18*)
Quartz	Human	+++	(*19,20*)
Crocidolite	Rat	+++	(*18,21*)
Crocidolite	Human	+++	(*20*)
Chrysotile	Rat	+++	(*17,21*)
Chrysotile	Human	+++	(*20*)
Coal dust	Human	++++	(*19*)
Titanium dioxide	Rat	–	(*18*)
Titanium dioxide	Human	+/–	(*19*)
Aluminum oxide	Rat	–	(*18*)
Latex beads	Rat	–	(*17*)

Modified from Driscoll et al. (*48*).[a]Relative responses were rated from – (no response) to +++++ (maximal response).

This paper is based on a presentation at The Sixth International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles held 15–18 September 1996 in Lake Placid, New York. Manuscript received at *EHP* 26 March 1997; accepted 18 July 1997.

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Abbreviations used: BAL, bronchoalveolar lavage; CINC, cytokine-induced neutrophil chemoattractant; DMSO, dimethysulfoxide; IκBα, inhibitory kappa B alpha; IL-8, interleukin-8; MCP-1, monocyte chemotactic protein 1; MIP-2, macrophage inflammatory protein-2; MMVF, man-made vitreous fiber(s); NF-κB, nuclear factor kappa B; TMTU, tetramethylthiourea; TNF-α, tumor necrosis factor alpha.

endotoxin as well as inflammatory mineral dusts such as quartz, crocidolite, and chrysotile, are potent agonists of alveolar macrophage TNF-α release, whereas the relatively innocuous materials titanium dioxide, aluminum oxide, and latex beads are not. The *in vivo* relevance of these *in vitro* findings on macrophage-derived TNF-α was later shown in studies demonstrating the increased production of TNF-α by alveolar macrophages from dust-exposed animals and humans. For example, intratracheal instillation of quartz particles into rat lungs was reported to markedly increase the release of TNF-α by alveolar macrophages. Similarly, increased steady-state TNF-α mRNA in rat lungs and immunoreactive TNF-α protein in rat alveolar macrophages were observed after prolonged inhalation of quartz or ultrafine titanium dioxide particles (*22*). In humans, Zhang and co-workers (*20*) demonstrated macrophage TNF-α production is elevated in asbestosis patients, as well as in individuals with idiopathic pulmonary fibrosis, an inflammatory lung disease.

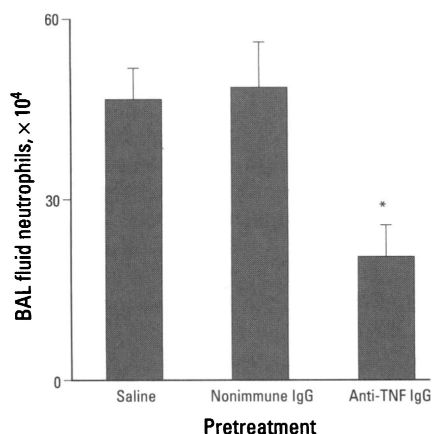

Figure 1. Passive immunization with anti-TNF-α IgG attenuates quartz-induced neutrophil recruitment in rat lungs. Groups of six F344 rats were injected via the tail vein with either 400 µl saline, nonimmune IgG (750 µg), or antimurine TNF-α IgG (750 µg) (Genzyme). Eighteen hours later, three animals with each pretreatment were exposed to air (control) or 100 mg/m^3 quartz for 6 hr. Twenty-four hours after quartz or air-sham inhalation exposure, the animals were sacrificed, the lungs were lavaged with saline, and the number of neutrophils in the BAL fluid was determined. No increase in neutrophils was observed for the air control groups. Quartz exposure resulted in a marked increase in BAL fluid neutrophil number; however, pretreatment with anti-TNF-α IgG significantly attenuated quartz-elicited increases in BAL fluid neutrophils; $p < 0.01$. Reproduced from Driscoll (*24*), with permission of CRC Press.

TNF-α is a multifunctional cytokine and its increased production within the lung may contribute to a variety of processes. There is evidence that one of the key actions of TNF-α is in the recruitment of inflammatory cells. Experimental evidence that TNF-α contributes to particle-elicited inflammation comes from studies demonstrating a significant correlation between *in vivo* exposure to particles and neutrophilic inflammation in rats. A similar correlation between neutrophilic inflammation and TNF-α in bronchoalveolar lavage (BAL) fluid has also been observed in humans (*20,23*). Recently, using a passive immunization approach, our laboratory provided more direct evidence for a role of TNF-α in particle-induced pulmonary inflammation (*22,24*). We treated mice intravenously with either a nonimmune IgG or an anti-TNF-α IgG and exposed the animals to an aerosol of quartz particles. As shown in Figure 1, neutrophilic inflammation characterized 24 hr after quartz inhalation was significantly reduced in animals pretreated with an antibody to TNF-α when compared to the nonimmune antibody treated controls. A similar antiinflammatory effect of passive immunization against TNF-α was demonstrated for the pulmonary inflammatory response of rats to intratracheally instilled titanium dioxide (*22*).

Chemokines

TNF-α itself is not chemotactic for neutrophils and macrophages. However, it can activate expression of several proteins that promote both adhesion of leukocytes and directed migration (i.e., chemotaxis) of these cells. This has led to the concept illustrated in Figure 2 that the mechanism by which TNF-α contributes to particle-induced inflammation is indirect and involves a network of cell:cell and cytokine interactions. Of particular importance with regard to chemotactic proteins is the ability of TNF-α to induce expression of members of a supergene family of cytokines known as chemokines. Members of the chemokine family possess a conserved four-cysteine motif, are heparin binding, and typically range in molecular weight from approximately 8 to 10 kDa.

The chemokine family can be subdivided into two branches based on structural and functional considerations (*25,26*). The C-X-C or α branch chemokines possess a structural motif in which the first two cysteines are separated by an intervening amino acid. The most well studied member of the C-X-C branch is IL-8, which exhibits both neutrophil and lymphocyte chemotactic activity. In the C-C or β branch of the chemokine family, the first two cysteines are adjacent. Members of this subgroup include monocyte chemotactic peptide-1 (MCP-1), macrophage inflammatory proteins (MIP)-1α and -1β, and RANTES. Functionally, most members of the C-C chemokine subgroup exhibit chemotactic activity for mononuclear cells with differing potencies for monocytes and various subpopulations of lymphocytes (*25*). Some members of the C-C branch can also attract and activate basophils and eosinophils (*27*). Recent

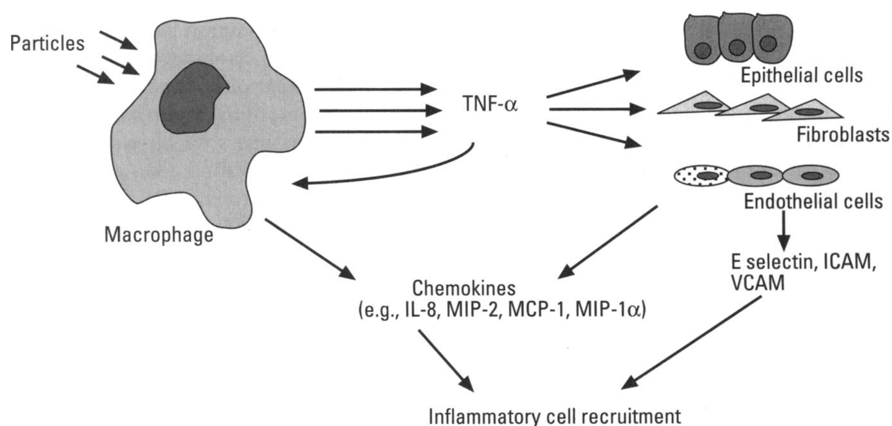

Figure 2. TNF-α-mediated cytokine networking and particle-induced inflammatory cell recruitment. Particles activate alveolar macrophages to release TNF-α, which acts via paracrine and autocrine pathways to stimulate release of chemokines by immune (e.g., macrophages) and nonimmune (e.g., epithelial cells: fibroblasts and endothelial) cells. Additionally, TNF-α increases expression of adhesion molecules (i.e., E selectin, ICAM, VCAM) on endothelial cells. Inflammatory cells interact with adhesion molecules on vascular endothelium and migrate along chemotactic gradients into the lung interstitium and airspaces. Modified from Driscoll (*24*).

studies indicate that MCP-1, RANTES, and MIP-1α can attenuate HIV infectivity *in vitro* (*28*).

A role for chemokines as mediators of particle-induced inflammation is suggested by studies demonstrating increased expression of these proteins in the lung after particle exposure. For example, increased macrophage production of TNF-α and lung expression of the gene for the chemokine MIP-2 (a neutrophil chemoattractant) was detected in rats within 24 hr after intratracheal instillation of inflammatory doses of quartz or titanium dioxide (*23,29*). Similarly, acute exposure of human volunteers to zinc oxide fumes resulted in increased BAL fluid TNF-α concentrations 3 hr after exposure. The increase in TNF-α was followed by increases in BAL fluid IL-8 concentrations 8 hr later (*30*), a response that was positively correlated with BAL fluid neutrophil numbers. More recently, subchronic inhalation exposure of rats to carbon black particles increased lung expression of chemokines MIP-2 and MCP-1 (a monocyte chemoattractant) (*31*). The increased chemokine expression in this inhalation study was associated with increased numbers of leukocytes in BAL fluid.

These studies imply a role for chemokines in particle exposure-elicited inflammation in the lung. Studies in rat lung provided more direct evidence that chemokines contribute to quartz-elicited neutrophilic inflammation and that TNF-α contributes to the quartz-induced increase in chemokine gene expression. Specifically, as shown in Figure 3, passive immunization of rats with an ip injection of rabbit anti-MIP-2 serum resulted in an approximate 60% reduction in inflammatory response to quartz instillation characterized by neutrophil numbers in BAL fluid (*32*). Considering a role for TNF-α in quartz-induced MIP-2 expression, using a similar passive immunization approach, it was shown that mice pretreated with an antibody to TNF-α exhibit a reduction in quartz-induced MIP-2 mRNA expression determined 6 hr after dust exposure (*33*). This observation is of particular importance, as it provides some of the first *in vivo* evidence that quartz-induced chemokine gene expression is dependent at least in part on TNF-α production.

Epithelial Cells and Particle-induced Chemokine Expression

As illustrated in Figure 2, one pathway by which inhaled particles may stimulate an inflammatory response is by activating macrophages to release TNF-α, which acts via autocrine or paracrine pathways to stimulate release of chemokines by cells such as macrophages, epithelial cells, and fibroblasts (*25,26,34,35*). An additional or alternate pathway by which inhaled agents may influence lung chemokine expression is through direct action on the lung epithelium. Like alveolar macrophages, epithelial cells are directly and continually exposed to the external environment; these cells respond to a diversity of stimuli *in vitro* with production of chemokines such as IL-8, MIP-2, cytokine-induced neutrophil chemoattractant (CINC), and MCP-1 (*29,34–36*). Thus, it is possible that particles could directly stimulate epithelial cells to release chemokines and stimulate inflammatory cell recruitment.

Evidence that epithelial cells may act as direct effectors of chemokine production in the lung after exposure to noxious particles has come from *in vitro* studies with human and rat alveolar epithelial cell lines and primary cultures of alveolar epithelial cells. Using human lung carcinoma cell line A549, Rosenthal et al. (*36*) investigated *in vitro* effects of the fibrogenic mineral fibers crocidolite and chrysotile on chemokine production. Both asbestos fibers increased mRNA for IL-8 and stimulated release of IL-8 protein by A549 cells, a response independent of TNF-α. Release of the chemokine MCP-1 was not affected by asbestos exposure, which indicated specificity in particle-induced effects on A549 chemokine expression. In contrast to crocidolite and chrysotile, exposure of A549 cells to the relatively less toxic particles wollastonite or titanium dioxide did not stimulate release of IL-8.

Recently our laboratory examined the ability of rat alveolar epithelial cells to produce chemokines in response to particles and other airborne agents with inflammatory activity. These studies were conducted using both the rat alveolar type II cell line RLE-6TN (*37*) and primary cultures of rat alveolar type II cells (*32*). Initial studies demonstrated that primary cultures of rat alveolar type II cells and the RLE-6TN cell line responded to TNF-α and bacterial endotoxin with increased steady-state levels of mRNA for MIP-2 and the functionally and structurally related chemokine CINC, but not the chemokine MIP-1α. These findings were consistent with earlier observations on the differential expression of MIP-2 and MIP-1α in nonleukocytic cells, and extended previous findings that production of IL-8 and MCP-1 is stimulated by TNF-α in A549 cells (*34,35*). These studies indicated that rat alveolar epithelial cells are potential sources of chemokines and that the RLE-6TN line may be a useful model to study rat alveolar epithelial responses.

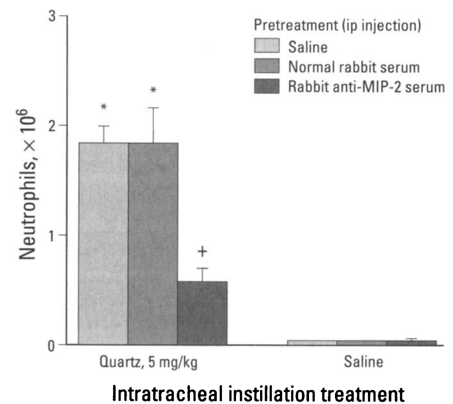

Figure 3. Effect of passive immunization of rats with anti-MIP-2 antiserum on quartz-elicited neutrophilic pulmonary inflammation. Rats were pretreated ip with saline, normal rabbit serum or rabbit antimurine MIP-2 antiserum, and IT instilled with saline or a saline suspension of 5 mg/kg quartz. Animals were sacrificed 24 hr after instillation and subjected to BAL, and the number of BAL fluid neutrophils was determined. Results represent the mean ± SD; $n = 3$ rats/treatment. Quartz exposure elici ted a significant neutrophilic inflammatory response. Pretreatment with anti-MIP-2 serum, but not nonimmune serum, significantly attenuated quartz-induced increases in BAL fluid neutrophils. *, statistically significant difference from the respective saline control group mean; $p < 0.05$. +, statistically significant difference from the respective saline control group mean and the other quartz-treated groups; $p < 0.05$. Reproduced from Driscoll et al. (*32*), with permission.

Previous studies demonstrated that quartz exposure of rats results in a rapid increase in mRNA for MIP-2 and that pretreatment of rats with antibody against MIP-2 attenuates quartz-induced pulmonary neutrophilic inflammation. To investigate the possibility that quartz may be increasing lung MIP-2 levels by acting directly on rat alveolar epithelial cells, we conducted a series of *in vitro* studies using RLE-6TN epithelial cells (*29*). As shown in Figures 4A and B, *in vitro* exposure to quartz increased expression of mRNA for MIP-2 and CINC in RLE-6TN cells. This response appeared dose related and was also observed in primary cultures of rat alveolar type II cells. Nuclear runoff analysis demonstrated that the quartz-induced increase in epithelial MIP-2 mRNA levels resulted, at least in part, from increased gene transcription (Figure 5), and was associated with release of immunoreactive and bioactive MIP-2 protein (*29*). No TNF-α was

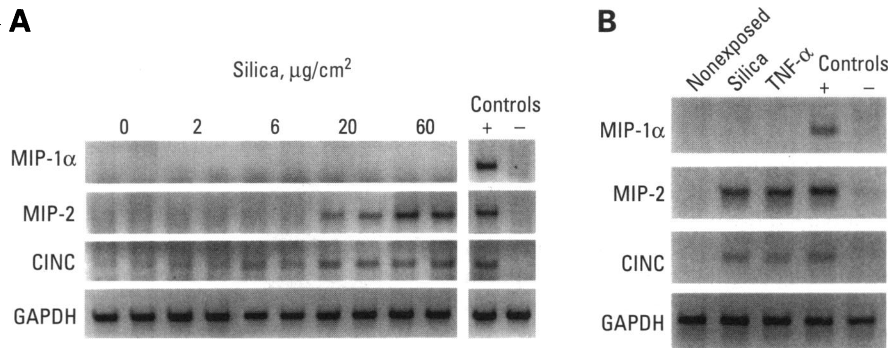

Figure 4. Expression of chemokine mRNA by rat lung epithelial cells after *in vitro* exposure to quartz. Epithelial cells were exposed *in vitro* to quartz particles or TNF-α as positive control. After a 6-hr exposure, RNA was isolated and reverse transcription–polymerase chain reaction (RT–PCR) performed to characterize steady-state levels of mRNA for chemokines MIP-1α, MIP-2 and CINC. Glyceraldehyde diphosphate dehydrogenase (GAPDH) was examined as control. 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 quartz on RLE-6TN cells. (*B*) Response of primary cultures of rat alveolar type II cells to quartz (20 µg/cm²) and TNF-α (50 ng/ml). Controls: +, total lung RNA from endotoxin-instilled rats; −, total lung RNA from untreated rats. Modified from Driscoll et al. (*32*).

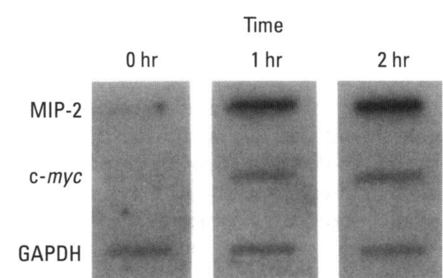

Figure 5. Transcriptional analysis of MIP-2, c-*myc*, and GAPDH genes in RLE-6TN cells exposed *in vitro* to 20 µg/cm², quartz for 0, 1, or 4 hr. ^{32}P-labeled RNA transcripts were hybridized with cDNA for MIP-2, c-*myc* and GAPDH immobilized on nitrocellulose, and radioactivity visualized by autoradiography. *In vitro* quartz exposure increased transcription of the MIP-2 gene and the c-*myc* gene in the rat alveolar epithelial cell lines RLE-6TN. Reproduced from Driscoll et al. (*32*), with permission.

detected in the epithelial cell cultures after exposure of the cells to quartz. These *in vitro* data strongly suggest that the induction of MIP-2 gene expression in rat lung epithelial cells after *in vivo* quartz exposure results from a direct effect of quartz on epithelial cells. Importantly, *in vitro* activation of epithelial cell MIP-2 gene expression is not unique to quartz but was not seen with the particles we examined. Like quartz, crocidolite asbestos fibers increased expression of MIP-2 mRNA in RLE-6TN cells. However, exposure to titanium dioxide or man-made vitreous fiber(s) (MMVF)10 glass fibers did not increase epithelial cell MIP-2 gene expression. The findings for crocidolite are consistent with those described for the A549 human lung carcinoma cell line (*36*). Like quartz, crocidolite fibers are highly inflammatory *in vivo* (*38,39*), whereas titanium dioxide particles and MMVF10 glass fibers are markedly less so (*40,41*). Thus, it is possible that epithelial cell chemokine release may be a factor contributing to the greater *in vivo* inflammatory activity of quartz and asbestos fibers.

Oxidants and Particle-activated Cytokine Expression

Increasing evidence suggests that oxidative stress exerted on a cell by particles may be an important factor influencing the expression of several proinflammatory and immunoregulatory cytokines. Simeonova and Luster (*21*) investigated the role of oxidants in chrysotile- and crocidolite-induced TNF-α production by rat alveolar macrophages. These investigators demonstrated that the induction of macrophage TNF-α gene expression and protein production by asbestos could be attenuated by the oxygen radical scavengers tetramethylthiourea (TMTU) and dimethylsulfoxide (DMSO), and stimulated by hydrogen peroxide or oxygen radical generating systems (e.g., xanthine–xanthine oxidase, glucose–glucose oxidase). It was also shown that iron was key in asbestos-induced effects, since iron chelators blocked the activation of macrophage TNF-α release. Similar studies suggest that oxidative stress contributes to quartz-induced epithelial cell expression of MIP-2. As illustrated in Figure 6A, we showed that the addition of TMTU to the RLE-6TN cell line attenuates quartz-induced MIP-2 gene expression; a similar result was observed when other antioxidant molecules (DMSO, ethanol, mannitol) were added to the cultures. In other studies, oxygen radical scavengers inhibited activation of IL-8 expression by

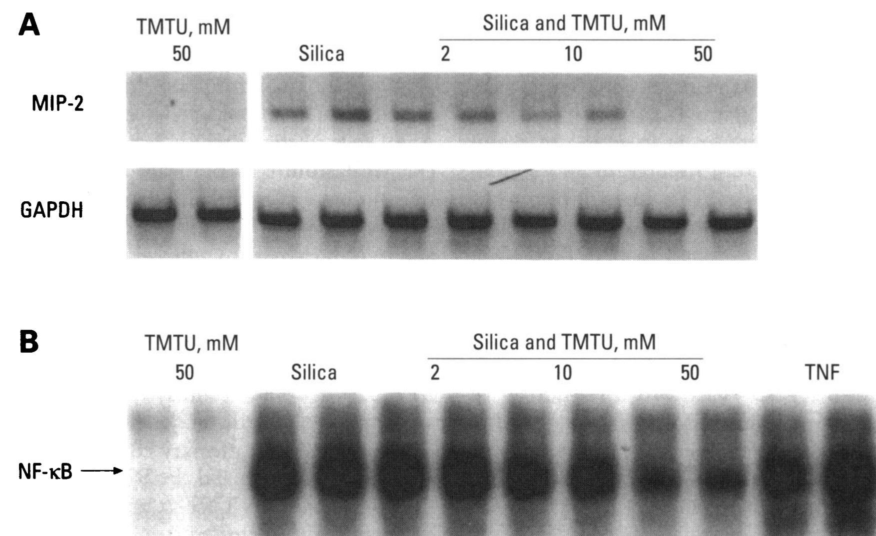

Figure 6. Effect of TMTU on silica-induced activation of MIP-2 gene expression and nuclear translocation of NF-κB in rat alveolar epithelial cells. The RLE-6TN rat alveolar epithelial cell line was exposed to silica (quartz) particles at 20 µg/cm² with and without TMTU. After a 6-hr exposure, RNA or nuclear proteins were isolated. RNA was used for analysis of MIP-2 gene expression using RT–PCR techniques. Nuclear proteins were used in a gel mobility shift assay to determined nuclear levels of NF-κB binding activity. (*A*) MIP-2 and GAPDH PCR products. Silica exposure increased levels of MIP-2 mRNA. Treatment with TMTU attenuated silica-induced increases in MIP-2 mRNA. (*B*) NF-κB nuclear binding activity. Quartz exposure increased nuclear levels of NF-κB binding activity. Treatment with TMTU attenua ted silica-induced increases in translocation of NF-κB.

human peripheral blood leukocytes (*42*). Overall, these findings indicate that oxidative stress plays a role in activating expression of some inflammatory cytokines after particle exposure.

The precise mechanisms by which particle-induced oxidative stress may activate cytokine gene expression are not known. However, it is noteworthy that the expression of several genes coding for inflammatory and immunoregulatory proteins are regulated by an oxidant-sensitive transcription factor known as nuclear factor kappa B (NF-κB). Included in this list are TNF-α, MIP-2, and IL-8 (*43–45*). NF-κB is a heterodimeric protein complex that in most cells resides in an inactive state in the cell cytoplasm. The NF-κB dimer is held in the cytoplasm by a protein known as inhibitory kappa B alpha (IκBα), which masks a nuclear translocation signal on NF-κB. Appropriate stimulation of a cell results in the phosphorylation of IκBα, signaling it for proteolytic degradation (*46*). Once IκBα dissociates from NF-κB, the latter can translocate into the nucleus and bind to appropriate sites in the DNA to influence transcription of various genes. While the signaling mechanisms contributing to the dissociation of IκBα and nuclear translocation of NF-κB have not been determined, this process can be stimulated by oxidants and inhibited by antioxidants (*45*). Regarding particle-stimulated cytokine gene expression, support for an oxidant dependent, NF-κB-mediated pathway in epithelial cells after quartz and crocidolite exposure has come from recent studies demonstrating increased nuclear translocation of NF-κB in hamster or rat lung epithelial cells exposed to these particles *in vitro* (*47,48*). Also supporting this pathway are preliminary results shown in Figure 6B, indicating that TMTU, which inhibits quartz-induced MIP-2 gene expression by RLE-6TN cells, also attenuates quartz-induced nuclear translocation of NF-κB in these cells.

Summary

There is evidence that in the lung, TNF-α plays a critical role in mediating inflammatory cell recruitment after exposure to a variety of particulate materials including inherently toxic particles such as quartz and crocidolite asbestos. Studies indicate that one pathway by which inhaled particles stimulate inflammatory responses is by activating alveolar macrophages to release TNF-α. TNF-α acts through a cytokine network and induces the expression of cytokines that are directly chemotactic for leukocytes and other cell populations as well as proteins that promote leukocyte adhesion. Effects of TNF-α can be mediated on both immune and nonimmune cells within the lung.

Chemokines are 6 to 15 kDa heparin-binding proteins that exhibit activities associated with inflammatory, immune, and tissue repair processes. Chemokines appear to be important contributors to inflammatory cell recruitment in the lung after particle exposure, and can be induced by TNF-α. Studies have implicated IL-8 in humans, and MIP-2 and CINC in rats, as mediators of particle-induced neutrophilic inflammation in the lung. In addition, particle exposure studies in rats suggest that MCP-1 may contribute to monocyte/macrophage recruitment.

Studies indicate that in addition to TNF-α-induced chemokines contributing to particle-induced inflammation, particles can activate lung epithelial cells directly to produce chemokines. These recent findings suggest that the direct activation of epithelial cells by inhaled materials may be an important additional or alternate pathway for initiating inflammatory responses in the lung. Investigators are beginning to understand the mechanisms by which inhaled particles activate epithelial cells to release certain chemokines. The expression of several inflammatory and immunoregulatory cytokines is regulated by the transcription factor NF-κB. Recent studies suggest that the ability of a particle to induce oxidative stress on a cell may be an important factor influencing its ability to activate the expression of cytokines such as TNF-α and MIP-2. Research in this area is beginning to define properties of particulate materials that contribute to their inflammatory activity in the lung.

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