Asbestos Induction of Nuclear Transcription Factors and Interleukin 8 Gene Regulation

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Proinflammatory cytokines and chemotactic peptides are strongly implicated as mediators of the pathophysiologic responses of asbestosis and other chronic inflammatory lung diseases. Recent studies in our laboratory have demonstrated that asbestos fibers stimulate lung epithelial cells to produce interleukin-8 (IL-8), the major neutrophil chemoattractant in the lung. The mechanisms by which asbestos regulates IL-8 expression were studied using the pulmonary type II–like epithelial cell line A549. Membrane permeable hydroxyl scavengers inhibited asbestos induced IL-8 expression. Using A549 cells transfected with the −546 IL-8 construct linked to a chloramphenicol acetyl transferase reporter gene, we have shown that these antioxidants directly inhibited asbestos-stimulated IL-8 promoter-dependent transcription. Asbestos fibers as well as reactive oxygen species generating systems hypoxanthine-xanthine oxidase and hydrogen peroxide stimulated DNA binding activity to the regulatory elements in the IL-8 promoter, binding sites of nuclear factor (NF)-κB– and NF-IL-6–like transcription factors. Asbestos-inducible DNA binding activity was partially inhibited by tetramethylthiourea, a hydroxyl radical scavenger. IL-8 secretion was also suppressed by staurosporine, an inhibitor of protein kinase C, and by inhibitors of tyrosine kinase such as herbimycin A and genistein. The suppression paralleled the effect of these inhibitors on asbestos-induced DNA binding to the NF-κB– and NF-IL-6–like binding sites of the IL-8 promoter. Taken together, the results suggest that asbestos-induced redox changes and phosphorylation events, mediated by staurosporine-sensitive and tyrosine kinase(s), activate nuclear proteins which recognize the NF-κB/NF-IL-6 binding sites of the IL-8 promoter and contribute to the regulation of IL-8 gene expression. **Simeonova, P. P., and M. I. Luster. 1996. Asbestos induction of nuclear transcription factors and interleukin 8 gene regulation. Am. J. Respir. Cell Mol. Biol. 15:787–795.**

Clinical and experimental evidence indicates that proinflammatory cytokines, chemotactic peptides, and growth factors from alveolar macrophages, lung fibroblasts and lung epithelial cells are important mediators in asbestos-related diseases (1–5). Recently, we demonstrated that the carcinogenic and fibrogenic asbestos fibers crocidolite and chrysotile directly stimulate the A549, human pulmonary type-II epithelial cell line, and primary human bronchial epithelial cells to express and secrete interleukin (IL)-8 in the absence of endogenous stimuli such as IL-1 and tumor necrosis factor α (TNF-α) (6). IL-8 secretion was not observed with nonfibrogenic particles such as titanium oxide. IL-8, a chemokine from the α-intecrine family, is one of the most stable and potent chemotactic and activating factors known for polymorphonuclear leukocytes (PMNs) and is produced by a variety of cell types including monocytes, epithelial, and endothelial cells (7–9). Alveolar neutrophil infiltration is a consistent observation in asbestos-exposed workers (10, 11) and has been implicated in the pathogenesis of asbestos-induced inflammation and fibrosis through the release of reactive oxygen species (ROS) and proteases with resulting tissue damage (12, 13).

Cytokine gene expression can be regulated at both the transcriptional and post-translational levels. Transcription is controlled by sequence-specific DNA binding proteins, referred to as transcription factors, which interact with gene's promoter or enhancer regions. NF-κB, a widely distributed

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Abbreviations: chloramphenicol acetyltransferase, CAT; 1,3-dimethyl-2-thiourea, DMTU; electrophoresis mobility shift assay, EMSA; glyceraldehyde 3-phosphate-dehydrogenase, G3PDH; hypoxanthine-xanthine oxidase, HX-XO; interleukin, IL; lactate dehydrogenase, LDH; *N*-acetylcysteine, NAC; nuclear factor, NF; hydroxyl radicals, ·OH; protein kinase A, PKA; protein kinase C, PKC; phorbol myristate acetate, PMA; reactive oxygen species, ROS; reverse transcription-polymerase chain reaction, RT-PCR; superoxide, O_2-; tyrosine kinase, TK; 1,1,3,3-tetramethyl-2-thiourea, TMTU; tumor necrosis factor α, TNF-α.

transcription factor, is involved in the regulation of genes encoding for many cytokines, including IL-8, IL-6, and TNF-α (14). For maximum expression of many of these cytokines, NF-κB binding must occur simultaneously with that of other transcription factors, such as NF-IL-6 (15, 16). In this respect, both NF-κB and NF-IL-6 binding sites are located at adjacent sites in the IL-8 promoter region (17). Furthermore, deletion analysis of the IL-8 promoter has indicated that both the NF-κB and NF-IL-6 binding sequences are necessary for the induction of IL-8 transcription by stimuli such as IL-1, TNF-α, or phorbol myristate acetate (PMA) (18, 19). DNA binding and transcriptional activation are regulated, at least in part, by post-translational modifications of the transcription factors. For example, oxidative stress can activate NF-κB by promoting IκB dissociation (20) and phosphorylation of NF-κB and NF-IL-6 precedes their binding to DNA cognate sequences and transcription activation (21, 22).

Asbestos fibers can stimulate cells to produce oxygen radicals and generate reactive species via iron that is present as a silicato-iron coordination complex on the fiber surface (23). Iron is a transition metal providing a source of electrons that catalyze the reduction of hydrogen peroxide (H_2O_2) and superoxide (O_2-), to generate hydroxyl ($\cdot OH$) radicals and other highly reactive oxidizing agents (24). Although reactive oxygen species (ROS) can play a direct role in asbestos-related damage, we recently demonstrated that ROS are also responsible for directly stimulating pro-inflammatory cytokine expression (25). Asbestos can also stimulate protein kinase C (PKC) activity (26) which, in turn, may initiate a cytokine response. As ROS and phosphorylation events have been implicated in the activation of transcription factors such as NF-κB, we hypothesized that the ability of asbestos fibers to activate IL-8 is via oxidative stress and/or PKC-mediated activation of NF-κB and NF-IL-6. Here, we demonstrate that asbestos activates IL-8 promoter-mediated transcription, as well as stimulates binding activity to the NF-κB/NF-IL-6 cognate regulatory elements in the IL-8 gene. The contribution of oxidative-redox changes and protein kinase activities were evaluated in the regulation of these responses.

Materials and Methods

Reagents

Crocidolite asbestos [$Na_2Fe_3Fe_2(Si_8O_{22})(OH)_2$], identified as ML-6 (Certain-Teed Co., Ambler, PA), was obtained from a mine in South Africa located in Kuruman Hills in the Kalahari desert. This sample of crocidolite had a mean length of 19 μm, a mean width of 1 μm and a density of 3.32 g/cm^3. Prior to use the mineral dust samples were sterilized and endotoxin inactivated by autoclaving for 2 h. There was no endotoxin detected in culture media incubated with the fibers as assessed by the Limulus amebocyte assay (Sigma Chemical Co., St. Louis, MO).

1,1,3,3-tetramethyl-2-thiourea (TMTU), 1,3-dimethyl-2-thiourea (DMTU), *N*-acetylcysteine (NAC), hypoxanthine (HX), xanthine oxidase (XO) (from buttermilk), and PMA were purchased from Sigma Chemical Co. Herbimycin A, H-89, genistein, staurosporine, and catalase (from bovine liver) were supplied from Calbiochem-Novabiochem Corp. (San Diego, CA). H_2O_2 was from Fisher Scientific Co. (Pittsburgh, PA) and dimethyl sulfoxide (DMSO) from J. T. Baker, Inc. (Phillipsburg, NJ).

Cell Preparation

Pulmonary human type II–like epithelial cells (A549), originally derived from an individual with alveolar cell carcinoma, were purchased from the American Type Culture Collection (ATCC; Rockville, MD). These cells retain many of the characteristics of normal type II cells, such as surfactant production, cytoplasmic multilamellar inclusion bodies, and cuboidal appearance, and have been extensively used to assess type II pneumocyte effector cell function (27). A549 epithelial cells were cultured in F12 media containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 2 mM glutamine, 50 μg/ml gentamicin, and 50 ng/ml fungizone (Gibco BRL, Gaithersburg, MD). Cultures were incubated overnight to achieve confluence and the test materials added in fresh medium. The cells were incubated for 18 h at 37°C and the supernatants were collected, filter sterilized, and stored at −70°C. Aliquots of cell culture supernatants were assayed immediately for the presence of lactic dehydrogenase (LDH), a measure of cytoplasmic leakage, as previously described (25). Triton X-100 [0.5% in phosphate-buffered saline (PBS)] was added for 30 min to control cultures to assess total LDH release.

TMTU, DMTU, and NAC were dissolved in culture media and added to the cells 1 h prior to treatment. The pH of NAC solution was adjusted to 7.2 by the addition of 2 N NaOH. Fresh solutions of H_2O_2 (1 M) were prepared in distilled water. All other chemicals were prepared fresh in PBS and added to cell cultures in 20-μl volumes.

IL-8 Enzyme-linked Immunosorbent Assay (ELISA)

Antibodies and recombinant human IL-8 were purchased from R&D Systems (Minneapolis, MN). Ninety-six-well Immulon plates (Dynatech Laboratories, Chantilly, VA) were coated with 2 μg/ml capture antibody (mouse monoclonal anti-human IL-8) in carbonate buffer (100 μl/well) for 24 h at 4°C. The plates were washed 5 times with PBS-0.5% Tween 20 solution (pH 7.3) and the free spaces were blocked by incubation for 2 h with 1% bovine serum albumin (BSA) in PBS-Tween 20. Samples or standards were added in 100 μl aliquots and incubated for 2 h at 37°C. The plates were rinsed and 100-μl of goat anti-human IL-8 polyclonal immunoglobulin G (IgG) antibody, diluted to 0.5 μg/ml, was added to each well for an additional 2 h. The plates were again washed and incubated with peroxidase-conjugate swine anti-goat IgG antibody, diluted 1:7,500, for 1 h. After washing, the wells were incubated with peroxidase substrate (H_2O_2/tetramethylbenzidine) for 20 min and the reaction was terminated by the addition of 50 μl of 2 N sulfuric acid. Absorbance was measured at 450 nM wavelength by using an ultraviolet (UV) max kinetic microplate reader (Molecular Devices, Corp., Menlo Park, CA) with the use of ΔSoft Program for data collection. IL-8 concentrations in test samples were determined from a standard curve of human recombinant IL-8. Data shown are representative of at least three separate experiments. Statistical significance was determined by the RS/1 Multicomparison procedure using Wilkes–

Shapiro test for normality, one-way analysis of variance (ANOVA), and Dunnett's test for multiple comparisons with a common control group (28). When variances were nonhomogeneous, multiple comparisons using the Bonferroni adjustment of the Student *t* test were performed. Statistically significant differences were reported when the *P* value was < 0.05.

Reverse Transcription and Polymerase Chain Reaction Amplification (RT-PCR)

Cellular RNA was extracted using RNAzol B (Biotec Laboratories, Inc., Houston, TX) according to manufacturer's instructions. For the synthesis of cDNA, 1 μg of RNA, quantitated spectrophotometrically (Gene Quant; Pharmacia, Cambridge, UK) was resuspended in a 20-μl final volume of the reaction buffer [25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM of each dNTP (Perkin Elmer, Cetus, Norwalk, CT)], containing 0.5 μg oligo d(T) 12-18 primer (BRL, Gaithersburg, MD). Once the reaction mixture reached 42°C, 400 U SuperScript (Gibco BRL; 200 U/μl) was added into each tube and incubated for 30 min at 42°C. The reaction was stopped by denaturating the enzyme at 99°C for 5 min and the mixture was diluted with water to 50 μl volume.

PCR amplification was performed using commercially available PCR primers (Clontech Laboratories, Inc., Palo Alto, CA) for human IL-8 and glyceraldehyde 3-phosphate-dehydrogenase (G3PDH), the latter an indicator of constitutive expression. The sequences of the primers used were as follows:

(*1*) IL-8 (sense: 5'-ATGACTTCCAAGCTGGCCGTGGCT3' antisense: 5'-TCTCAGCCCTCTTCAAAAACTTCTC3')
(*2*) G3PDH (sense: 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3', antisense: 5'-CATGTGGGCCATGAGGTCCACCAC-3')

Five-microliter aliquots of the synthesized cDNA (corresponding to approximately 0.1 μg RNA) were added to 45 μl of PCR mix containing 4 μl of 10× PCR buffer, 1 μl deoxynucleotides (1 mM each), 1 μl of 3' and 5' RNA-specific primers sense and anti-sense primers (0.15 μM) and 0.25 μl DNA polymerase (Gene Amp PCR kit; Perkin Elmer Cetus, Norwalk, CT). The reaction mixture was covered with Ampli (Gem) wax tablets (Perkin Elmer Cetus). Amplification was initiated by 1 min of denaturation at 94°C for one cycle followed by 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min using a GeneAmp PCR 9600 DNA Thermal Cycler (Perkin Elmer Cetus). After the last cycle of amplification, the samples were incubated for 7 min at 72°C. For each set of primers, serial dilutions of cDNA were amplified at increasing cycles (25 to 30) to achieve the optimal conditions for amplification. The PCR products were visualized by UV illumination following electrophoresis through 2.0% agarose (UltraPure; Sigma) at 60 V for 80 min and staining in Tris borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2.5% EDTA, pH 8.2) containing 0.5 mg/ml ethidium bromide. The relative amount of mRNA transcripts was determined visually and by laser densitometry. When necessary the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA, in which fragments were generated of the expected size (data not shown).

Quantitative analysis of cytokine message was conducted by means of competitive PCR using stepwise dilutions with a synthetic competitor (PCR MIMICS; Clontech Laboratories, Palo Alto, CA) as a template. The competitors are sequences complementary to the cytokine primers but with different size PCR products. Three-microliter aliquots of cDNA were amplified for 25 to 30 cycles with the cytokine primers in the presence of the respective competitor, ranging from 10^2 to 10^{-5} attomoles of competitor. The relative amount of mRNA transcripts was determined visually and by laser densitometry. The ratio of the target densitometry value was plotted against the reciprocal of the molar amount of the competitor.

Nuclear Extracts and Electrophoresis Mobility Shift Assay (EMSA)

Nuclear proteins were prepared from aliquot of 1×10^7 cells according to the method of Dignam and colleagues (29). DNA binding reactions and EMSA were performed as described previously (30). Briefly, the 5' ends of the double-stranded oligonucleotides were labeled with γ^{32}P-adenosine triphosphate (New England Nuclear/Dupont, Boston, MA), using 6 to 10 U of T4 polynucleotide kinase (USB/Amersham, Cleveland, OH). Ten micrograms of nuclear proteins was incubated on ice in 30 μl of buffer composed of 20 mM Tris-HCl (pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 50 μg/ml BSA, 2 μg of poly(dI-dC) · poly(dI-dC), 10% glycerol, and approximately 0.1 ng (2×10^5 cpm) of specified probe. The sequences of the oligonucleotides used as probes or competitors have been described (17, 19) and are as follows. κB-NF-IL-6 (-99/-66 human IL-8, containing binding sites for NF-IL-6 and NFκB): 5'-CCATCAGTTGCAAATCGTGGAATTTCCTCTGACA-3'; AP-1 (-131/117 human IL-8 gene, containing binding site for AP-1): 5'-GTGATGACTCAGGTT-3'; NF-κB (-85/-68 human IL-8 gene, containing binding site for NF-κB): 5'-TCGTGGAATTTCCTCTGA-3'; NF-IL-6 (-99/-78 human IL-8 gene, containing binding site for NF-IL-6): 5'-CCATCAGTTGCAAATCGTGGAA-3'; NF-IL-6 binding site in human IL-6 gene: 5'-GGACGTCACATTGCACAATCTTAATAA-3'; NF-κB binding site in human IL-6 gene: 5'-TTATCAAATGTGGGATTTTCCCATGAGTCTCAAT-3'.

Protein–DNA complexes were separated on a 5% nondenaturing polyacrylamide gel. Gels were electrophoresed at 125 V in 50 mM Tris-50 mM boric acid/1 mM EDTA, dried, and autoradiographed overnight. For characterization of DNA binding activity, the nuclear protein extracts were preincubated prior to the addition of labeled probe with an excess of unlabeled oligomers for 30 min, or with 5 μg of specific antibodies to the NF-κB and NF-IL-6 subunits (Santa Cruz Biotech., Inc., Santa Cruz, CA) for 1 h.

Cell Transfections and Chloramphenicol Acetyltransferase (CAT) Assay

The IL-8 construct pUXCAT -546 IL-8 (31), used for cell transfections, was a gift from Dr. N. Mukaida, of the Cancer Research Institute, Kanazawa University, Japan. A549 cells (2×10^5 cells/well) were seeded into 35-mm tissue culture

plates in F12 culture medium supplemented with 10% FBS and 50 μg/ml gentamicin and allowed to grow until they reached 60 to 70% confluence. Transfections were carried out with lipofectamine (Gibco BRL), according to the manufacturer's instructions. The transfection mixture (total volume 0.2 ml) contained 2 μg of total plasmid DNA (reporter gene expression vector and β-actin luciferase, LUC vector used as an internal control) and 6 μl of lipofectamine in serum- and antibiotic-free F12 culture medium. After incubation for 5 h at 37°C in 95% air/5% CO_2, the medium was replaced with 2 ml of fresh F12 medium containing insulin-transferrin-selenium supplement (Gibco BRL) and the cells were exposed to test samples for 18 h. CAT levels were determined quantitatively by a commercial ELISA (Boehringer Mannheim, Indianapolis, IN). Cell extracts were prepared using a lysing buffer (Boehringer Mannheim) and luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Protein concentrations were determined using a commercially available protein assay system (BioRad, Hercules, CA).

Results

Initially, studies were undertaken to determine whether oxidative stress was involved in IL-8 production in lung epithelial cells by asbestos. As shown in Figure 1a, the addition of intracellular OH· radical scavengers, including DMTU, TMTU, or DMSO to cell cultures stimulated with asbestos fibers effectively decreased IL-8 secretion. Inhibition of IL-8 by antioxidants was dose-dependent as exemplified by a representative response curve conducted with TMTU (Figure 1b). Although asbestos causes some cytotoxicity, inhibition of cytokine release by antioxidants occurred in the absence of changes in cytotoxicity, as determined by lactate dehydrogenase (LDH) release (Figure 1a and b, *inset*). To examine whether inhibition of IL-8 secretion was reflected at the level of gene expression, RNA was isolated from control and asbestos-treated A549 cultured in the presence or absence of TMTU and cytokine mRNA transcripts quantitated by competitive RT-PCR (Figure 1c). The constitutively expressed gene, G3PDH, was not affected by asbestos or by antioxidants and was used to adjust for equal cDNA concentrations. Based on linear regression analysis of densitometric values, it was estimated that asbestos treatment produced an increase in IL-8 mRNA transcripts approximating several orders of magnitude, whereas in the presence of TMTU the response was similar to the control levels. The ability of ROS to stimulate IL-8 secretion in A549 cells was further demonstrated by the radical generating system of hypoxanthine-xanthine oxidase (HX-XO) or H_2O_2 (Figure 2). Treatment with either H_2O_2 or HX-XO induced a significant increase in IL-8 secretion compared with untreated cells. The increased IL-8 secretion was mediated by oxidative stress as heat-inactivated XO, which prevents the production of radicals, or addition of catalase to H_2O_2-inhibited cytokine production.

To determine whether oxidative stress directly stimulates the IL-8 promoter, transient transfections of A549 cells were conducted using the IL-8 construct (pUXCAT-546), which contains binding sites for several transcription factors, in-

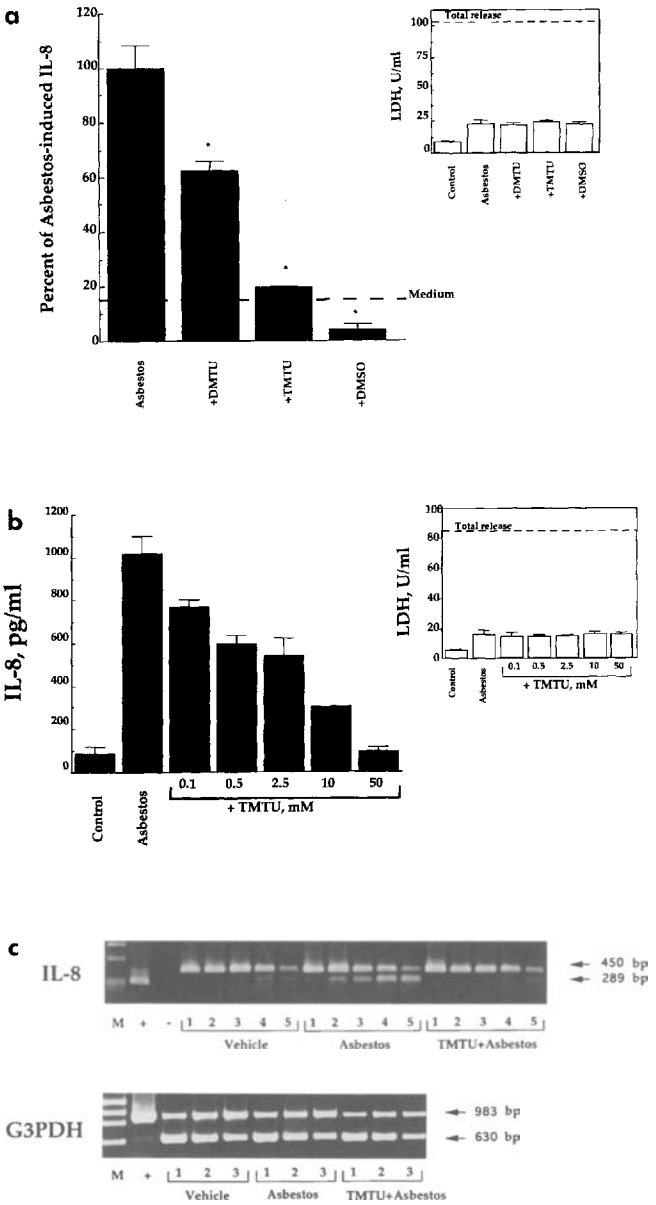

Figure 1. Effect of antioxidants on asbestos-induced IL-8 response in A549 lung epithelial cells. (*Panel a*) Cells were pretreated for 1 h with 10 mM DMTU or TMTU, or 1% DMSO, and exposed to asbestos (25 μg/ml). The ELISA values are presented as the percentage of asbestos-induced IL-8 secretion. *Asterisk* represents a significant ($P < 0.05$) decrease from the response of cells treated only with asbestos fibers. LDH release was used as a measure of cellular damage. Values represent the mean ± SE of three cultures. (*Panel b*): Dose response of TMTU on asbestos-stimulated IL-8 and LDH secretion. Values represent the mean ± SE of three experiments. (*Panel c*): Quantitative analysis of IL-8 and G3PDH mRNA levels from A549 cells cultured for 2 h in medium, asbestos (25 μg/ml), or asbestos (25 μg/ml) plus TMTU (10 mM) by competitive RT-PCR. cDNA equivalents of 0.1 μg RNA were amplified for 28 or 25 cycles in the presence of 3-fold dilutions of IL-8 or G3PDH competitors, respectively. For IL-8: top band, IL-8 competitor; bottom band, sample IL-8. Lane 1, 0.250 attomoles competitor; lane 2, 0.083 attomoles; lane 3, 0.028 attomoles; lane 4, 0.009 attomoles; lane 5, 0.003 attomoles. For G3PDH: top band, sample G3PDH; bottom band, G3PDH competitor. Lane 1, 20 attomoles; lane 2, 6.6 attomoles; lane 3, 2.2 attomoles.

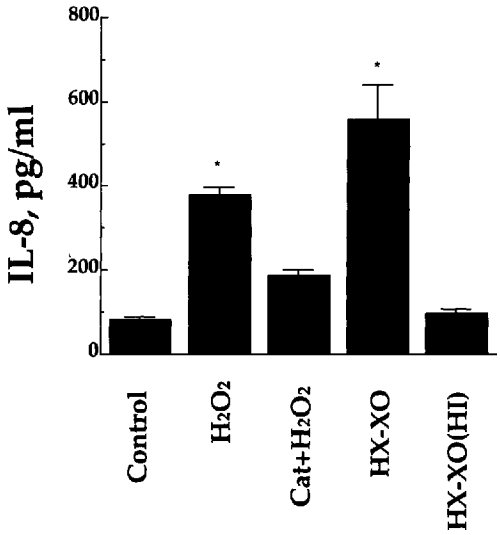

Figure 2. IL-8 secretion from A549 cells exposed to H_2O_2 (0.5 mM) or HX (0.5 mM)-XO (10 mU). The specificity of reaction was examined by addition of catalase (Cat; 100 U/ml) prior to treatment with H_2O_2, or the use of heat-inactivated (HI; 10 min at 70°C) XO. Values represent the mean ± SE of three experiments. *Asterisk* represents statistically ($P < 0.05$) different from untreated cells.

cluding NF-κB and NF-IL-6 (31). CAT expression was increased approximately 3-fold following incubation of transfected cells with asbestos fibers (Figure 3). Actin-luciferase construct activity, which was not affected by asbestos exposure, was used to normalize for transfection efficiency. The increase in CAT expression in the presense of asbestos was prevented completely by the addition of TMTU and reduced significantly in the presence of DMTU. Neither antioxidant affected CAT expression in control cultures.

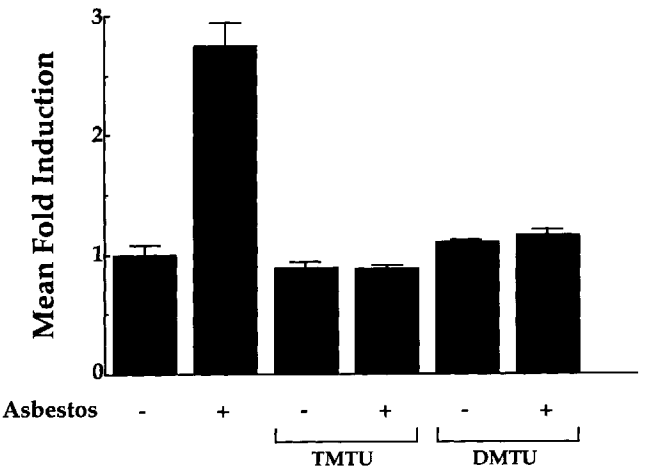

Figure 3. Inhibition of asbestos-induced, IL-8 promoter-driven transcription by antioxidants. A549 cells were transfected with 1.5 μg of IL-8 (-546)-CAT reporter plasmid and 0.5 μg of β-actin LUC control plasmid. The transfected cells were cultured with medium alone, asbestos (12.5 μg/ml), asbestos in the presence of 10 mM TMTU or DMTU, or only TMTU or DMTU. The activation of IL-8 promoter (-546) was determined by assaying the CAT concentration of cellular extracts prepared 26 to 28 h after transfection. Transfection efficiencies were normalized by analyzing LUC activity. The results are presented relative to CAT levels in unstimulated cells. Experiments were repeated 3 times with representative data shown.

Mobility shift assays were conducted to characterize asbestos-induced nuclear protein-DNA binding activity to the IL-8 promoter regulatory elements which are associated with IL-8 expression. A -99/-66 fragment from the IL-8 gene, containing the adjacent NF-IL-6- and NF-κB-like binding sites, was used to probe nuclear extracts from A549 cells. As with IL-8 gene expression, DNA binding activity was present constitutively in A549 cells and markedly increased following incubation with asbestos (Figure 4a). Binding of both the constitutive and asbestos-inducible complexes was blocked by addition of excess unlabeled oligonucleotide competitor containing the NF-IL-6/NF-κB-like binding sites, but not by an unrelated sequence containing the AP-1 binding site. Additionally, competitive binding studies with oligonucleotides containing binding sites only for NF-κB- or NF-IL-6-like transcription factors demonstrated that constitutive and asbestos-induced DNA binding were dependent on both NF-κB- and NF-IL-6-like binding sites (Figure 4a). Asbestos-inducible binding activity was not demonstrated when the extracts were probed with the -99/-66 oligonucleotides with mutations in the NF-κB or NF-IL-6 binding sites (data not shown). Asbestos-induced nuclear proteins recognizing NF-κB- and NF-IL-6-like binding sites were characterized immunochemically using antibodies against several known members of the NF-κB and NF-IL-6 families of transcription factors (Figure 4b). Asbestos activates the DNA binding of both the p50 and p65 subunits of the NF-κB transcription factor, as the complex was supershifted by anti-p50 antibody and abrogated by anti-p65 antibody. Asbestos-induced nuclear protein binding to the binding site of NF-IL-6, a member of C/EBP family of transcription factors, was supershifted by anti-C/EBPβ (NF-IL-6) but was not affected by anti-C/EBPα or C/EBP δ antibodies.

The ability of ROS to stimulate binding activity to the NF-κB/NF-IL-6-recognized sequence (-99/-66) in the IL-8 gene was examined by EMSA. Nuclear extracts from A549 cells cultured with H_2O_2 or HX-XO, revealed an increase in DNA binding of 2- and 2.5-fold, respectively (Figure 5a). Addition of TMTU to cell cultures stimulated with asbestos reduced inducible binding activity by 50% (Figure 5b).

In addition to ROS, protein kinases have been reported to regulate gene expression by altering NF-κB and NF-IL-6 DNA binding activities. To assess the potential role of kinases in the asbestos-induced IL-8 response, various protein kinase inhibitors were added to cell cultures and IL-8 secretion monitored. The IC_{50} values, derived from regression analysis of the reduction in IL-8 secretion, are shown in Table 1 and are compared with the published inhibitory constants (K_i) of the various inhibitors. Staurosporine, an inhibitor of several serine-threonine kinases, including PKC, dose dependently inhibited IL-8 secretion by asbestos with an IC_{50} of 0.05 μM. This was comparable to the IC_{50} obtained for IL-8 secretion following stimulation with PMA. Relatively high concentration of H89 was required to reduce IL-8 secretion by asbestos ($IC_{50} > 23$ μM), suggesting that its effect may not be specific for PKA. Herbimycin A, a

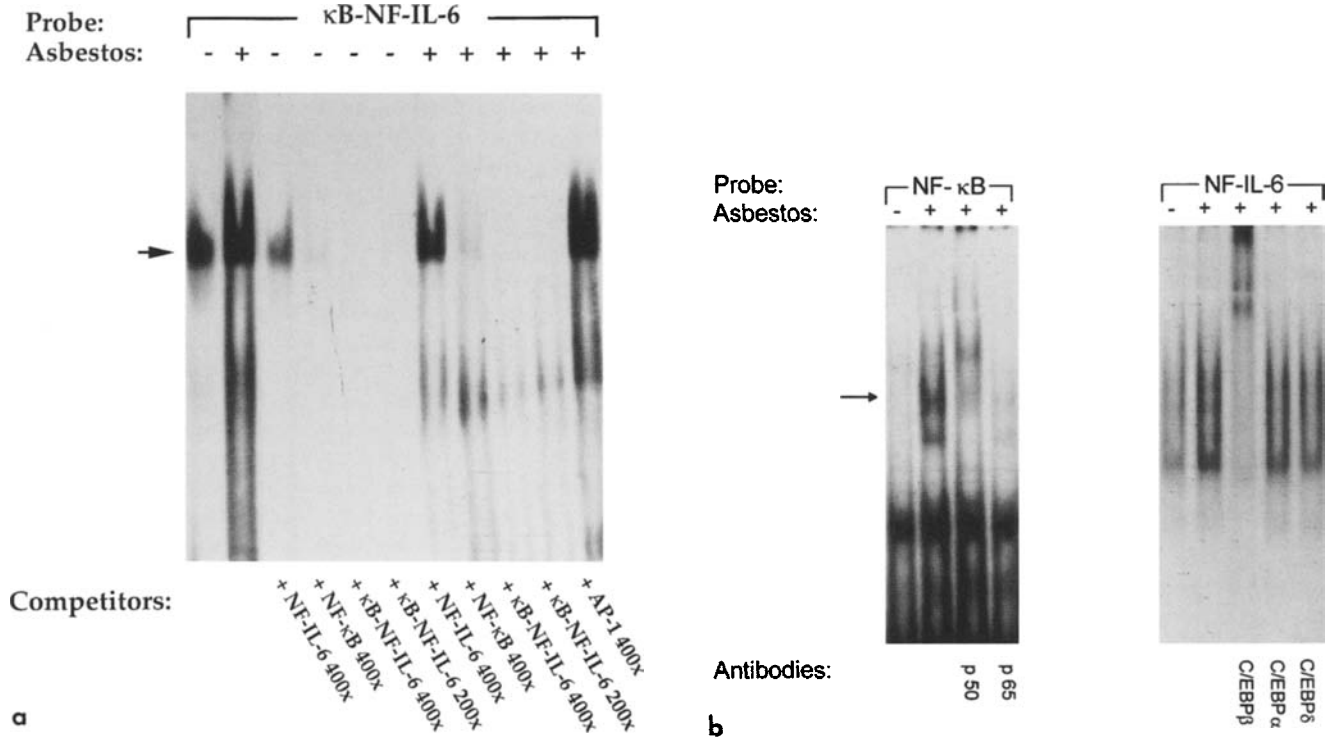

Figure 4. Characterization of asbestos-induced DNA binding activity to the NF-IL-6– and NF-κB–like binding sites. Nuclear extracts from control or asbestos (25 μg/ml; 2 h)-stimulated A549 cells were analyzed by EMSA. The *arrow* indicates a specific band. (*Panel a*): The ^{32}P-labeled -99/-66 sequence of the IL-8 gene, which contains binding sites for NF-κB/NF-IL-6 was used as a probe, and 200× or 400× excess of the unlabeled oligonucleotides, binding sites for κB-NF-IL-6, NF-κB, NF-IL-6, or AP-1 were used as competitors. (*Panel b*): Nuclear extracts were preincubated with 5-μg antibodies to p50, p65, or C/EBPα, C/EBPβ, and C/EBPδ, respectively, and probed with ^{32}P-labeled NF-κB–like or NF-IL-6 binding sites from IL-6 promoter.

specific inhibitor of PTK, caused significant inhibition of IL-8 secretion at low concentrations (IC_{50} > 2.0 μM). Genistein, a PTK inhibitor with less specificity than herbimycin A, also effectively suppressed asbestos-induced IL-8 (IC_{50} > 33.5 μM). The protein kinase inhibitors did not produce cytotoxicity at their IC_{50} concentrations when assessed by LDH release (data not shown). Taken together these results suggest that staurosporine-sensitive and tyrosine kinase(s) may be associated with asbestos signaling of the IL-8 response. The effects of staurosporine, herbimycin A, and genestein on DNA binding were characterized further by EMSA using the -99/-66 sequence of the IL-8 gene (Figure 6). Similar to IL-8 secretion, staurosporine prevented asbestos-inducible nuclear protein–DNA binding to the NF-κB– and NF-IL-6–like binding sites in the IL-8 promoter. Herbimycin A and genestein were less effective under these conditions and reduced asbestos-inducible binding activity by over 50%.

Discussion

Asbestos exposure in humans is associated with pulmonary interstitial fibrosis characterized by the accumulation of inflammatory cells within the lung, followed by the progressive deposition of extracellular matrix and subsequent destruction of the lung airspaces (32). The release of pro-inflammatory cytokines, chemotactic peptides, and growth factors is strongly implicated in the pathophysiology of asbestos-induced pulmonary diseases (1–5). Historically, alveolar macrophages have been considered responsible for the generation of inflammatory mediators in the lung. However, since the alveolar space is lined by epithelial cells, direct asbestos–epithelial contact also occurs, resulting in the secretion of cytokines and chemokines which participate in

TABLE 1

IL-8 inhibition by protein kinase inhibitors (PKI)

Inhibitors	Specificity of PKI K_i (μM)* PKC	PKA	Stimulus TK	IC_{50} (μM)†
Staurosporine	0.0007	0.007	0.3	Asbestos; PMA > 0.05
Herbimycin A	NA	NA	0.9	Asbestos > 2.0
Genistein	400	400	2.6	Asbestos > 33.5
H89	32	0.05	NA	Asbestos > 23.4

* Reference/K_i values for protein kinase C (PKC), protein kinase A (PKA), or tyrosine kinase (TK) (Calbiochem Signal Transduction Catalog and Technical Resource, 1995).

† IC_{50} value (concentration which induces 50% inhibition of the stimulated IL-8 secretion) calculated by regression analysis of IL-8 dose-response inhibition. NA = not available.

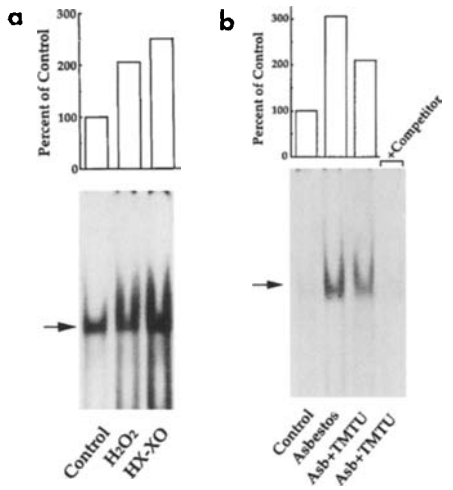

Figure 5. Effect of oxidative stress on the binding activity of nuclear factors from A549 cells to the NF-IL-6/NF-κB-like binding sites in the IL-8 promoter. The ^{32}P-labeled -99/-66 sequence of the IL-8 gene was used as a probe. The results are quantified by radioactivity image analysis and presented as a percentage of the response by unstimulated cells. (*Panel a*): EMSAs were conducted with nuclear extracts from cells exposed for 2 h to H_2O_2 (0.5 mM) or HX (0.5 mM)-XO (10 mU). (*Panel b*): EMSAs with nuclear extracts from cells exposed to asbestos (25 mg/ml) or asbestos plus TMTU (10 mM) (12 h pretreatment). A 100× excess of the unlabeled oligonucleotides κB-NF-IL-6 was used as a competitor.

asbestos-induced chronic inflammation. In this respect, chronic hyperplasia and hypertrophy of type II cells occur in animal models of asbestosis (33), and we previously have reported that IL-8 is readily induced by asbestos in A549 and

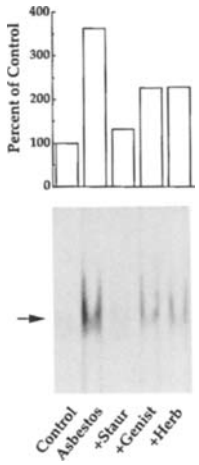

Figure 6. Effect of protein kinase inhibitors on asbestos-induced binding activity to the NF-IL-6/NF-κB-like binding sites in the IL-8 promoter. Nuclear extracts were prepared from A549 cells pretreated for 2 h with staurosporine (Staur; 0.1 μM), genistein (Genist; 74 μM), or herbimycin A (Herb; 3.4 μM) and stimulated by asbestos (25 μg/ml) for an additional 2 h. The ^{32}P-labeled -99/-66 sequence of the IL-8 gene was used as a probe. The results are quantified by radioactivity image analysis and presented as a percentage of the response of unstimulated cells.

primary human bronchoepithelial cells without the generation of primary-response cytokines such as IL-1 or TNF-α (6). As IL-8 is the major neutrophil chemotactic and activating factor in the lung (7), and the presence of neutrophils is a frequent finding in animal models for asbestosis (33) as well as humans exposed occupationally (10, 11), it can be hypothesized that the presence of IL-8 is associated with immunopathologic processes in asbestosis. Elevated numbers of PMNs in bronchial alveolar lavage fluid (BAL) and increased levels of IL-8 are also prominent features in idiopathic pulmonary fibrosis (IPF), adult respiratory distress syndrome, cystic fibrosis, and chronic bronchitis (34–36). Lung damage associated with PMN activation is due, in large part, to the generation of ROS and the release of proteolytic enzymes which can destroy the respiratory structure and subsequently impair pulmonary function. The present studies suggest that asbestos fibers induce IL-8 secretion and expression in parallel with activation of nuclear protein binding to the regulatory elements in the IL-8 gene recognized by NF-κB- and NF-IL-6–like transcription factors. Asbestos-induced oxidative stress, resulting from Fenton-type reactions, is likely involved in regulating IL-8 gene expression as cellular redox changes modulate nuclear protein DNA binding activities to the NF-κB and NF-IL-6 cognate elements in the IL-8 promoter. Tyrosine-dependent and staurosporine-sensitive phosphorylation events, including NF-κB- and NF-IL-6–like nuclear protein activation, also appear to play a role in stimulating IL-8 gene expression.

There is increasing evidence that NF-κB activation is an oxidative stress-responsive transcription factor and ROS serves as secondary messengers (20). For example, H_2O_2 can activate NF-κB DNA binding activity in several cell lines, while antioxidants prevent this activation (37). It has been postulated that ROS cause IκB dissociation resulting in release of the activated NF-κB complex. Reactive oxygen metabolites, such as OH· radicals, have also been implicated as important mediators in asbestos-induced lung injury (23), and we have previously provided evidence that ROS generated from asbestos–cell interactions stimulates expression of inflammatory cytokines (25). Consistent with the present observation, it has been recently demonstrated that asbestos fibers can activate NF-κB in hamster tracheal epithelial cells (HTE) as a result of glutathione level modulation (38). We observed that the expression and secretion of IL-8 by asbestos in A549 pulmonary epithelial cells are inhibited by hydroxyl radical scavengers. The participation of ROS in IL-8 secretion was confirmed by the use of free radical generating systems including H_2O_2 and HX-XO. The ability of ROS to stimulate IL-8 has also been demonstrated in whole blood following *in vitro* stimulation with LPS, PHA, or H_2O_2 as well as in several cell model systems (39). Furthermore, using A549 cells transfected with an IL-8 (-546)-CAT plasmid, asbestos-induced oxidative stress was demonstrated to directly activate the IL-8 promoter. Similar to asbestos fibers, the oxidative state induced by HX-XO or H_2O_2 stimulates DNA binding to the NF-κB- and NF-IL-6–like binding sequences in the IL-8 promoter region. In contrast to the total inhibition observed in the transfection assay, the redox changes in the presence of intracellular antioxidants did not abrogate the binding of the nuclear transcription factors. TMTU only partially reduced asbestos-induced bind-

ing to NF-κB/NF-IL-6–like sites on the IL-8 promoter. This suggested that oxidative stress modulates IL-8 expression not only by activation of nuclear transcription factor binding, but also by interactions between activated transcription factors and events downstream. In this respect, it has been reported that INF-β inhibition of TNF-α–induced IL-8 gene expression is mediated by NF-κB elements on the IL-8 promoter, but without altering the NF-κB DNA binding ability (31).

The 5′-flanking region of the IL-8 gene contains binding sites for several transcription factors including AP-1, AP-2, hepatocyte nuclear factor 1, interferon regulatory factor 1, glucocorticoid response element, as well as NF-κB and NF-IL-6 (17). Experimental data suggest that different families of transcription factors (e.g., NF-κB, NF-IL-6, AP-1) and the interactions among them allow for the varied, coordinated regulation of IL-8 gene expression which will vary based on both the cell type and the inducing agent (15, 40). Mukaida and colleagues (15), reviewing the data on IL-8 transcriptional regulation, suggested that while NF-κB is central for IL-8 gene transcription, cooperation with other transcription factors, particularly NF-IL-6, is required for optimal expression. Studies with protein members of the C/EBP and NF-κB families of transcription factors indicated that functional as well as direct protein–protein interactions occur between these factors as a result of the presence of a basic leucine-zipper (bZIP) region and Rel homology domain (18, 19, 41). Asbestos fibers induced DNA binding activity to the IL-8 gene segment (-99/-66), which includes both NF-κB–like and NF-IL-6–like binding sites. Both sites in the IL-8 promoter sequence were necessary to display the binding activity of A549 cell nuclear proteins. These nuclear proteins bind probably integrated, as the binding complex was not affected following preincubation of the nuclear extracts with specific antibodies to NF-κB and C/EBP proteins (data not shown). Some of these antibodies under identical conditions identified asbestos-induced nuclear proteins, bound to the NF-κB and NF-IL-6 recognized sites in IL-6 promoter, where the NF-κB binding site is located 70 bp downstream to the NF-IL-6 binding site.

IL-8 secretion by asbestos was suppressed by staurosporine, an inhibitor of PKC, as well as PTK inhibitors such as herbimycin A and genistein. The concentrations of staurosporine required to prevent IL-8 secretion are consistent with those that would inhibit PKC but as well as other sensitive kinases, such as protein kinase G and PKA, indicating a lack of specificity at these concentrations. As the dose-response was analogous to staurosporine inhibition of IL-8 produced by PMA, an activator of PKC, and asbestos crocidolite activates PKC in hamster tracheal epithelial cells (HTE) (26), a role of PKC in signaling IL-8 can be considered. Staurosporine also totally blocked nuclear protein binding to the NF-κB/NF-IL-6 sites in the IL-8 gene. Herbimicyn A and genistein were effective inhibitors of IL-8 secretion and reduced nuclear protein binding to the NF-κB/NF-IL-6–like binding sites in IL-8 promoter at concentrations consistent with their inhibitory constants for tyrosine kinases. In contrast, H89 did not inhibit IL-8 at concentrations specific for PKA. Thus, both tyrosine kinase and PKC phosphorylation contribute to the activation of transcription factors, which recognize regulatory elements in the IL-8 promoter sequence (-99/-66). Increasing evidence indicates that NF-κB activation is associated with phosphorylation of its members (21). Recent studies have demonstrated that staurosporine and tyrosine kinase inhibitors block LPS-induced binding activity to the NF-κB–like site in the IL-8 gene (42). The binding potential of C/EBP transcription factors may also be regulated by phosphorylation events (22).

In conclusion, asbestos fibers were shown to induce IL-8 gene expression and secretion in A549 cells, a prototype of human pulmonary type II epithelial cells, and this response is mediated by asbestos-activated oxidative stress. Redox changes are involved in the control of IL-8 expression as well as in the promoter activation. Phosphorylation events induced by tyrosine kinases and staurosporine-sensitive kinases are also an important step in the regulation of asbestos-induced IL-8 expression. Further investigations of these molecular mechanisms should lead to a better understanding of the pathogenesis of fiber-induced lung diseases and development of novel therapeutics.

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