Critical role of glass fiber length in TNF-α production and transcription factor activation in macrophages

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Ye, Jianping, Xianglin Shi, William Jones, Yon Rojanasakul, Ningli Cheng, Diane Schwegler-Berry, Paul Baron, Gregory J. Deye, Changhong Li, and Vincent Castranova. Critical role of glass fiber length in TNF-α production and transcription factor activation in macrophages. *Am. J. Physiol.* 276 (*Lung Cell. Mol. Physiol.* 20): L426–L434, 1999.—Recent studies have demonstrated that dielectrophoresis is an efficient method for the separation of fibers according to fiber length. This method allows the investigation of fiber-cell interactions with fiber samples of the same composition but of different lengths. In the present study, we analyzed the effects of length on the interaction between glass fibers and macrophages by focusing on production of the inflammatory cytokine tumor necrosis factor (TNF)-α in a mouse macrophage cell line (RAW 264.7). The underlying molecular mechanisms controlling TNF-α production were investigated at the gene transcription level. The results show that glass fibers induced TNF-α production in macrophages and that this induction was associated with activation of the gene promoter. Activation of the transcription factor nuclear factor (NF)-κB was responsible for this induced promoter activity. The inhibition of both TNF-α production and NF-κB activation by *N*-acetyl-L-cysteine, an antioxidant, indicates that generation of oxidants may contribute to the induction of this cytokine and activation of this transcription factor by glass fibers. Long fibers (17 μm) were significantly more potent than short fibers (7 μm) in inducing NF-κB activation, the gene promoter activity, and the production of TNF-α. This fiber length-dependent difference in the stimulatory potency correlated with the fact that macrophages were able to completely engulf short glass fibers, whereas phagocytosis of long glass fibers was incomplete. These results suggest that fiber length plays a critical role in the potential pathogenicity of glass fibers.

nuclear factor-κB; free radicals; tumor necrosis factor-α

FIBROUS MATERIALS have various applications in both residential and industrial settings. These materials offer, in varying degrees, reinforcement, thermal and electrical insulation, flexibility, and strength. Asbestos is one group of such materials that exhibits these properties. Animal experiments and epidemiologic studies have concluded that asbestos exposure is associated with various lung diseases including fibrosis and cancer (23). As a result of these findings, the use of asbestos has been limited or prohibited in several countries. Consequently, the development and use of new fibers are on the increase. These include different types of man-made materials such as fibrous glass, rock wool, and ceramic fibers. Although these fibers are believed to be less toxic than asbestos, only limited studies (1, 9, 13, 14, 16, 21, 32, 34) of their toxic effects exist. The mechanisms of toxic and biological actions are not fully known. Two studies (14, 32) indicated that their chemical composition can affect the ability of fibers to generate toxic oxidants, which can damage the lung cells. For example, transition metals on the fiber surface can catalyze the generation of reactive oxygen species (32). Recent studies (10, 17, 22) also indicated that fiber length seems to be an important factor. For example, when implanted in the pleural spaces of rats, long asbestos fibers exhibited a higher carcinogenic activity than short fibers (30). The amount of fibers deposited in the lung is dependent on the concentration, size, shape, and other physical properties of the fibers (14, 33). It has been postulated that alveolar deposition decreases with increasing fiber diameter and length (14). Once in the lung, the biological activity of the inhaled fibers is dependent on the length, the physical and chemical properties of the surface (14), and the solubility of the fibers. Although the above studies point to an important role for fiber length in toxicity, pertinent studies have not been undertaken to support this hypothesis. This is likely due to the difficulty of generating fibers with well-defined lengths in quantities sufficient to carry out laboratory studies. Recently, a classifier has been developed to separate fibers by length with dielectrophoresis that involves the movement of neutral particles in a gradient electric field (5). The development of this classifier makes it possible to study the role of fiber length in toxicity. Exploring the ability of this technique, Blake et al. (7) recently studied the toxicity of JM-100 glass fibers and found that long fibers were more toxic than short fibers when dose was expressed on a fiber-count basis.

The present study focuses on the length dependence of induction of tumor necrosis factor (TNF)-α production and nuclear transcription factor (NF)-κB activation in a macrophage cell line (RAW 264.7). TNF-α was chosen because this inflammatory and fibrogenic cyto-

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kine is a macrophage-derived peptide that has been shown to play an important role in the pathogenesis of pulmonary fibrosis (15, 25, 40). Several fibrogenic agents, such as crystalline silica and asbestos, stimulate TNF-α mRNA expression and protein synthesis in macrophages (25, 40). Elevated TNF-α levels lead to fibrosis by stimulating proliferation of fibroblasts and production of collagen matrix (8, 25, 40). Cytokine gene expression can be regulated at both the transcriptional and posttranscriptional levels. Transcription of TNF-α is controlled by sequence-specific transcription factors, including NF-κB, that interact with the gene promoter or enhancer regions. NF-κB, a widely distributed multisubunit transcription factor, is involved in the regulation of genes encoding for many cytokines (12). In the present study, we attempted to answer the following questions. *1*) Do glass fibers induce TNF-α production in vitro? *2*) Do glass fibers cause NF-κB activation in vitro? *3*) Does fiber length play a critical role in TNF-α production or NF-κB activation? *4*) Does NF-κB regulate TNF-α production induced by glass fibers? *5*) Are reactive oxygen species involved in TNF-α production or NF-κB activation? *6*) Do long fibers interact differently with macrophages than short fibers?

MATERIALS AND METHODS

Fibers. Bulk samples of Manville code 100 (JM-100) glass fibers supplied by the manufacturer were first milled, aerosolized, and separated into two length categories by dielectrophoresis (5). The dielectrophoretic classifier was operated in a differential mode so that fibers with narrow length distributions were extracted in an air suspension at the end of the classifier. These size-selected fiber samples were collected on polycarbonate (Nuclepore) filters at rates of up to 1 mg/day. Fibers were scraped off the filters for microscopic analysis and biological experiments.

We prepared samples of the length-classified fibers for size and count analysis by adding weighed portions of the dusts to freshly filtered water. These samples were then sonicated, diluted, and filtered through polycarbonate filters. Measurements of length, width, and fiber count per mass were made with a JEOL JSM-6400 scanning electron microscope. Measurements at each magnification were referenced to a National Institute of Standards and Technology electron microscopy standard rule.

In this study, glass fiber samples with lengths (means ± SD) of 7 (6.5 ± 2.7 μm) and 17 μm (16.7 ± 10.6 μm), respectively, were used to analyze the effects of fibers on macrophages. Concentrations of the glass fibers used in these experiments were determined as fiber counts per milliliter. The glass fiber counts per milligram are 3.0×10^8 and 2.0×10^7 for 7- and 17-μm fiber samples, respectively (7). The glass fiber samples were heat treated at 120°C for 2 h and stored under sterile conditions at room temperature. Before each experiment, the glass fibers were suspended and sonicated in the complete cell culture medium and then applied to cells.

The endotoxin content of the glass fiber samples was measured with the *Limulus* amebocyte lysate assay (24). Values ranged from 0.7 to 1.69 endotoxin units (EU)/mg. These values are orders of magnitude lower than those found with cotton dust (1,000–2,000 EU/mg) or agricultural dusts (46–4,000 EU/mg) where endotoxin is thought to play a role. The maximum dose of glass fiber used in this study is 700 μg/ml, in which the maximum endotoxin concentration equals 0.118 ng/ml. This dose of endotoxin had no effect on the RAW 264.7 cells because the minimum effective dose of endotoxin is 1 ng/ml (data not shown). Therefore, the fiber results reported here cannot be attributed to endotoxin contamination.

Cells and reagents. The mouse monocyte-macrophage cell line RAW 264.7 was purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in complete medium containing DMEM supplemented with 10% FCS, 2 mM glutamine, and 100 U/ml of penicillin-streptomycin. A specific antibody against the NF-κB p50 subunit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used in the supershift assay. *N*-acetyl-L-cysteine (NAC) was purchased from Sigma (St. Louis, MO). These reagents were freshly prepared in a phosphate-buffered solution (PBS buffer) as a 20-fold stock solution and kept at 4°C. SN50 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA).

TNF-α ELISA assay. The macrophage cells (1×10^5/well) were plated in 96-well plates for 4–16 h before stimulation. NAC or SN50 was preincubated with the cells for 30 min to inhibit fiber-induced TNF-α production or NF-κB activation. Three wells were used in each treatment. The cell culture supernatant was harvested at the end of treatment, combined together from the three wells, and used for the TNF-α assay. An ELISA kit from Genzyme (Cambridge, MA) was used to determine TNF-α production according to the manufacturer's instructions.

Gel shift assay. An NF-κB binding sequence in the human interleukin (IL)-6 gene promoter (bases −74 to −54, TGGGATTTTCCCATGAGTCT) was used to synthesize an oligonucleotide for the NF-κB binding probe (18). The complementary single-stranded oligonucleotides were denatured at 80°C for 5 min and annealed at room temperature. An activator protein (AP)-1 binding oligonucleotide derived from the AP-1 binding sequence in the collagenase gene promoter was used as a nonspecific competitor or as a probe to examine AP-1 binding activity (36, 39). The double-stranded probe was labeled with [^{32}P]ATP (Amersham, Arlington Heights, IL) with the T4 kinase (Bethesda Research Laboratories, Gaithersburg, MD). The nuclear extracts were prepared with a three-step procedure. First, the harvested cells were treated with 500 μl of lysis buffer (50 mM KCl, 0.5% Nonidet P-40 (NP-40), 25 mM HEPES, pH 7.8, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, 20 μg/ml of aprotinin, and 100 μM 1,4-dithiothreitol) on ice for 4 min. The cell lysate was centrifuged at 14,000 rpm for 1 min, and the supernatant was discarded. Second, the collected nuclei were washed once in a washing buffer that had the same composition as the lysis buffer without NP-40. Third, the nuclei were treated with an extraction buffer (500 mM KCl and 10% glycerol with the same concentrations of HEPES, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and 1,4-dithiothreitol as the lysis buffer) to make the nuclear extract. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at −70°C. The protein concentration was determined with a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). The DNA-protein binding reaction was conducted in a 24-μl reaction mixture including 1 μg of poly(dI-dC) (Sigma), 3 μg of nuclear protein extract, 3 μg of BSA, 4×10^4 counts/min of ^{32}P-labeled oligonucleotide probe, and 12 μl of reaction buffer (24% glycerol, 24 mM HEPES, pH 7.9, 8 mM Tris·HCl, pH 7.9, 2 mM EDTA, and 2 mM 1,4-dithiothreitol) (37). In some cases, the indicated amount of double-stranded oligomer was added as a cold competitor. The reaction mixture was incubated on ice for 10 or 20 min (with antibody) in the absence of radiolabeled probe. After the addition of the radiolabeled

probe, the mixture was incubated for 20 min at room temperature, then resolved on a 5% acrylamide gel that had been prerun at 170 V for 30 min with 0.5× Tris-borate-EDTA buffer. The loaded gel was run at 200 V for 90 min, then dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY). The film was developed after an overnight exposure at −70°C.

Transfection assay. The reporter gene vector used in this study was a gift from Dr. S. T. Fan (Scripps Research Institute, La Jolla, CA) (35). The luciferase vector contains a promoter fragment (bases −615 to +15) of the human TNF-α gene. The murine macrophages (1×10^6/well) were plated in six-well plates for 16 h before transfection. The reporter DNA (5 μg) was delivered into the cells by the DEAE-dextran method (35). After transfection, the cells were washed once in PBS solution and cultured in 3 ml of the complete medium at 37°C for 24 h. After being stimulated for an additional 16 h, the cells were harvested for the reporter assay. The luciferase activity was determined with an assay kit (Promega, Madison, WI), then normalized for the protein content.

Data analysis. Data that are reported as means ± SD of three individual experiments were analyzed by Student's *t*-test at a confidence level of $P < 0.05$–0.001.

RESULTS

Time course of TNF-α production by macrophages in response to glass fiber stimulation. The murine RAW 264.7 cell line was used as a model to study TNF-α production by macrophages exposed to size-classified samples of glass fibers. We examined the stimulatory activities of the glass fibers by exposing macrophages to samples of short (7-μm) or long (17-μm) glass fibers. The concentration of glass fibers is expressed as the ratio of fiber count to cell count. These fiber concentrations did not cause cytotoxicity as measured by trypan blue exclusion (data not shown). TNF-α production by macrophages was determined by an ELISA assay. We conducted the time-course study by stimulating cells with glass fibers at a 5:1 ratio (fiber to cell) for 3, 6, or 16 h. The glass fibers failed to induce TNF-α production after a 3-h exposure. However, a significant induction of TNF-α was observed after exposure for 6 or 16 h (Fig. 1). The short and long fibers exhibited a significant difference in the ability to induce TNF-α protein. The long-fiber samples (17-μm length) exhibited a significantly stronger stimulation of TNF-α production compared with the short-fiber samples (7-μm length) at both the 6- and 16-h incubation points.

Dose-dependent stimulation of TNF-α production by glass fibers. According to the result of the time-course study, a 16-h exposure of macrophages to glass fibers caused the greatest stimulation of TNF-α production. Therefore, this exposure time was used to examine the dose dependence of glass fibers on TNF-α production. Macrophages were exposed to the short or long glass fibers at fiber-to-cell ratios over the range of 0 to 30 as indicated in Fig. 2. A significant induction of TNF-α production was observed at or above a fiber-to-cell ratio of 5:1 for both short and long glass fiber samples. However, long fibers generated a significantly greater stimulation than short fibers at ratios of 5:1 and above, exhibiting 2.5- to 3-fold higher TNF-α production at each exposure dose.

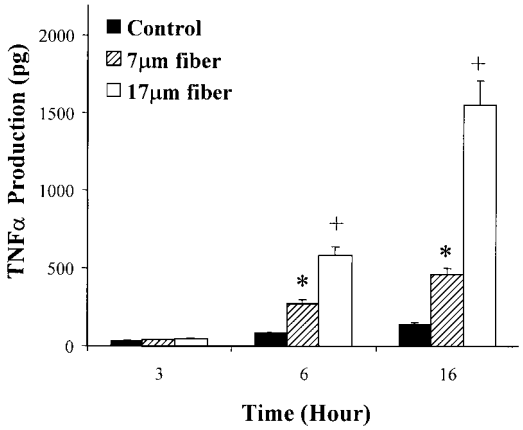


Fig. 1. Time course of tumor necrosis factor (TNF)-α production in RAW 264.7 cells exposed to glass fibers. Cells were treated with fibers at fiber-to-cell ratio of 5:1 for different times. TNF-α production was determined with an ELISA assay as stated in MATERIALS AND METHODS. Data are means ± SE of 3 independent experiments. *Significant increase in production of TNF-α induced by short fibers compared with untreated cells, $P < 0.001$. $^+$Significant increase in TNF-α production induced by long fibers compared with short fibers, $P < 0.001$.

Activation of TNF-α gene promoter by glass fibers. Cytokine gene expression is controlled at multiple levels, including transcription, RNA stability, and translation. It is well known that transcriptional regulation is a key step in the control of TNF-α gene expression. The gene promoter activity of TNF-α was thus examined to investigate the mechanism of glass fiber activation of macrophages. A luciferase reporter vector that is controlled by a wild-type human TNF-α gene promoter was used to analyze the transcriptional activity under glass fiber stimulation. Macrophages were transfected by the plasmid vector and then exposed to size-selected glass fiber samples. The reporter assay indicates that the TNF-α gene promoter was activated after the transfected cells were exposed to either the short or long glass fibers (Fig. 3). However, compared with the

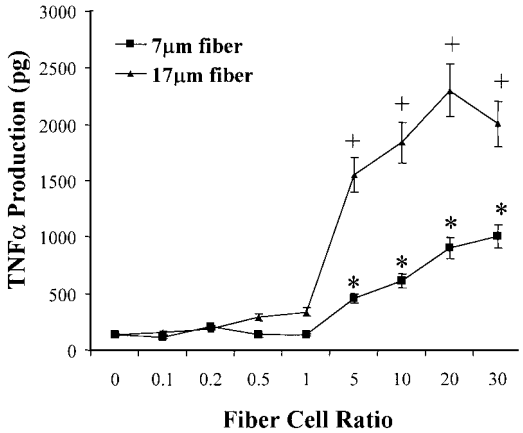


Fig. 2. Dose-response relationship of glass fibers. Different fiber-to-cell ratios were used to treat cells in culture plate for 16 h. TNF-α production was determined by ELISA assay. *Significant increase in production of TNF-α induced by short fibers compared with untreated cells, $P < 0.001$. $^+$Significant increase in TNF-α production induced by long fibers compared with short fibers, $P < 0.001$.

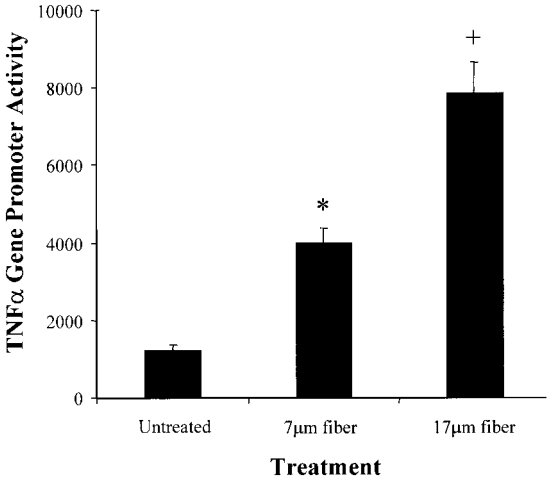


Fig. 3. Activation of TNF-α gene promoter by glass fibers. Transient transfection assay was used to study gene promoter activity of TNF-α. Luciferase reporter controlled by a TNF-α gene promoter was transfected into cells. Transfected cells were treated with fibers for 16 h at fiber-to-cell ratio of 5:1. Reporter activity in cell lysate was determined with a luminometer, and reading was normalized for amount of protein used in each reporter assay. Data are means ± SD of reporter activity from 3 independent assays. *Significant increase in induction of promoter activity by short fibers compared with untreated cells, $P < 0.001$. $^{+}$Significant increase in induction of promoter activity induced by long fibers compared with short fibers, $P < 0.001$.

short fibers, the long fibers exhibited a 100% increase in the stimulatory activity.

Induction of DNA binding activity of the transcription factor NF-κB by glass fibers. NF-κB is a ubiquitous transcription factor that plays an important role in the control of gene expression of many cytokines. The requirement of NF-κB activation in the induction of TNF-α gene promoter activity has been well established in the signaling pathway in macrophages (12). Because the TNF-α gene promoter is activated by the exposure of macrophages to glass fibers, NF-κB might be a mediator in the signaling pathway of glass fibers. The DNA binding activity of NF-κB was investigated in the nuclear extracts of macrophages with the gel shift assay. The results show that a significant induction of DNA binding was detected in the nuclear extract of macrophages treated with either short or long fibers (Fig. 4, *A* and *B*). The long fibers expressed stronger activity than the short fibers. The DNA-NF-κB complexes usually generated two bands in the gel. The upper band was formed by the p50/p65 heterodimer, and the lower band was formed by the p50/p50 homodimer. Because the upper and lower bands were both induced, it indicated that the p65 and p50 subunits of NF-κB were all activated. The RAW 264.7 cell nuclear protein formed a typical pattern of NF-κB bands with the radiolabeled probe. This was confirmed with oligonucleotide competition and antibody supershift studies, although only p50 antibody was used (Fig. 4*C*). Unlabeled NF-κB probe effectively competed with the radiolabeled probe in NF-κB protein binding, whereas unlabeled AP-1 probe had no effect. The antibody against the p50 subunit of NF-κB shifted the p50/p50 band and reduced the p50/p65 band, whereas antibodies for SP-1 or AP-1 had no effect. The results support the conclusion that NF-κB is involved in the signaling pathway after glass fiber exposure. DNA binding activity of AP-1 was also investigated, and it was induced by glass fibers (data not shown).

Inhibition of NF-κB and TNF-α gene promoter activities by NAC and SN50. The data in *Induction of DNA binding activity of the transcription factor NF-κB by glass fibers* indicate that NF-κB is activated in macrophages exposed to glass fibers, but its activation may not be necessary for the activation of TNF-α gene transcription because many transcription factors are involved in the regulation of this gene promoter. If the promoter requires the transcription factor, inhibition of NF-κB activity should result in a suppression of TNF-α gene promoter activity. NAC and SN50 were used to examine the role of NF-κB in the glass fiber-induced promoter activity. NAC is an established antioxidant that has been shown to block NF-κB activation induced by reactive oxygen species (4, 29). SN50 is a small peptide containing a cell membrane-permeable amino acid sequence and a translocation signal of the NF-κB p50 subunit (20). This agent can specifically block translocation of NF-κB from the cytoplasm into the nucleus. Both NAC and SN50 effectively inhibited NF-κB activation in macrophages exposed to long fibers (Fig. 5, *A* nd *B*). Under the same conditions, the TNF-α gene promoter was also significantly inhibited (Fig. 5*C*). Taken together, these results suggest that activation of NF-κB is required for the activation of the TNF-α gene promoter in macrophages exposed to glass fibers. The toxicity of both inhibitors was examined with the dose used in this study, and no significant change in cell viability was observed in all experiments.

Suppression of glass fiber-induced TNF-α production by NAC and SN50. The results in *Inhibition of NF-κB and TNF-α gene promoter activities by NAC and SN50* show that glass fibers activate TNF-α gene expression at the transcriptional level. Is this a major mechanism of glass fiber activity? If so, inhibition of this promoter activity should lead to a similar inhibition of TNF-α secretion. To examine this hypothesis further, TNF-α production by macrophages was monitored after addition of NAC or SN50. Figure 6 shows that these inhibitors dramatically block TNF-α production in response to both short and long glass fibers. This result supports the conclusion that transcriptional regulation is a major mechanism of gene expression and protein production induced by glass fibers.

Engulfment of the glass fibers by macrophages. Interaction between glass fibers and macrophages is the cause of activation of NF-κB and the TNF-α gene. It is of interest to explore how macrophages respond morphologically to a challenge with glass fibers of different lengths. Therefore, macrophages were observed under light microscopy after exposure to short or long glass fibers for 16 h. The short and long fibers were handled differently by the cells. Short fibers (7 μm) were effectively engulfed by the macrophages (Fig. 7*A*). In con-

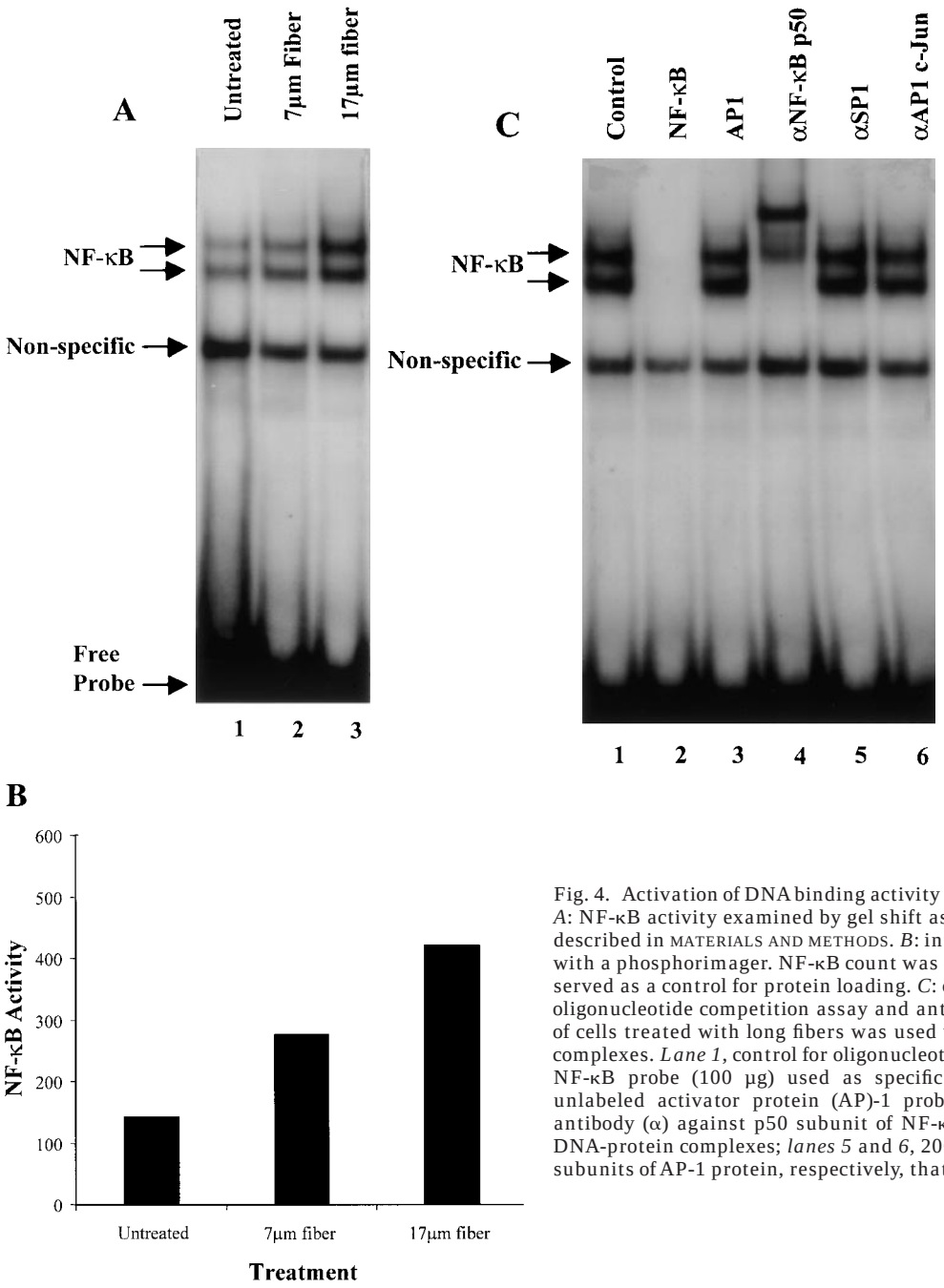
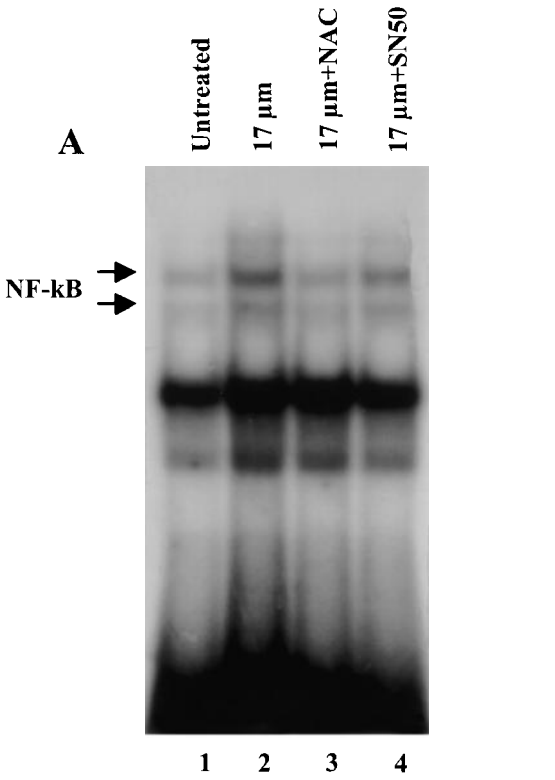


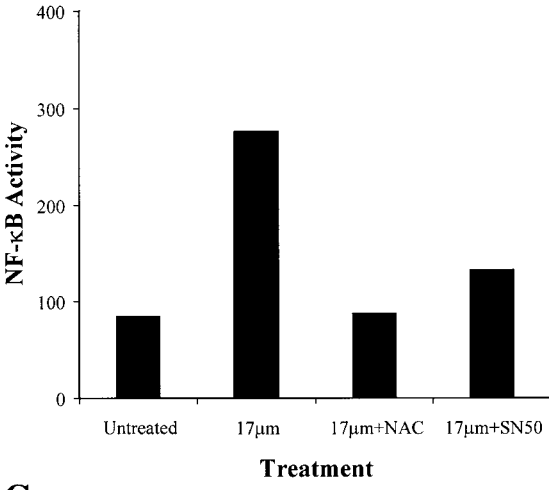
Fig. 4. Activation of DNA binding activity of nuclear factor (NF)-κB by glass fibers. *A*: NF-κB activity examined by gel shift assay after cells were exposed to fibers as described in MATERIALS AND METHODS. *B*: intensity of NF-κB bands in *A* quantitated with a phosphorimager. NF-κB count was divided by a nonspecific band count that served as a control for protein loading. *C*: characterization of NF-κB complexes by oligonucleotide competition assay and antibody supershift assay. Nuclear protein of cells treated with long fibers was used with NF-κB probe to form DNA-protein complexes. *Lane 1*, control for oligonucleotide competition assay; *lane 2*, unlabeled NF-κB probe (100 μg) used as specific competitor; *lane 3*, same amount of unlabeled activator protein (AP)-1 probe for nonspecific competition; *lane 4*, antibody (α) against p50 subunit of NF-κB protein to confirm protein nature of DNA-protein complexes; *lanes 5* and *6*, 200 μg of antibody against SP-1 and c-Jun subunits of AP-1 protein, respectively, that served as nonspecific antibodies.

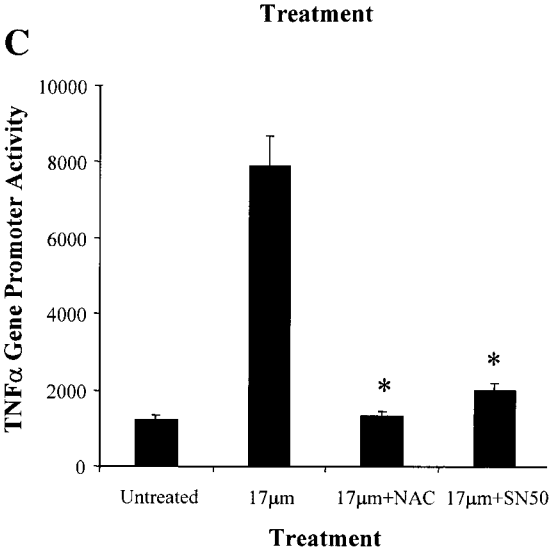
trast, long fibers (17 μm) were not completely phagocytized (Fig. 7*B*). Many long fibers remained extracellular. A common feature is that both short and long fibers induced cell fusion and formation of giant cells containing two or more nuclei in this macrophage cell line. This was observed after cellular staining (data not shown). Such cell fusion was not observed with primary rat alveolar macrophages (7).

DISCUSSION

The present study examines TNF-α production and transcription factor activation in macrophages after exposure to glass fibers and investigates the critical role of fiber length. The results indicate that at subtoxic doses, glass fibers stimulate TNF-α production in a macrophage cell line (RAW 264.7). These fibers also cause NF-κB activation. Inhibition of NF-κB by NAC, an antioxidant that inhibits NF-κB activation induced by reactive oxygen species or SN50, a specific NF-κB inhibitor, suppressed the promoter function of the TNF-α gene and decreased TNF-α secretion by macrophages, demonstrating that TNF-α production was regulated by NF-κB. Inductions of NF-κB, promoter activity, and TNF-α protein do not have to have the same multiple of change. NF-κB signal may be amplified in the gene promoter, and the signal may be further amplified in the process of mRNA and protein synthesis. The amplification in the posttranscriptional part may not be dependent on the activation of NF-κB. A signal from NF-κB is required for the initiation of







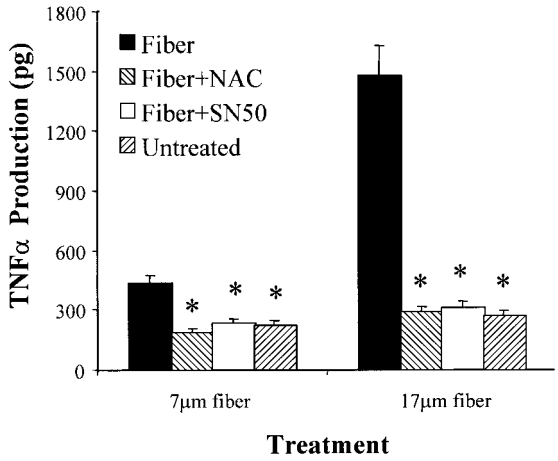


Fig. 6. Inhibition of fiber-induced TNF-α production by NAC and SN50. NAC (20 mM) and SN50 (100 μg/ml) were used to treat cells in presence of short and long fibers. TNF-α production was determined after 16 h of combined treatment. Data are means ± SD of TNF-α levels from 3 independent experiments. *Significant suppression of TNF-α production, $P < 0.001$.

TNF-α gene transcription induced by glass fibers. Functions of the NF-κB complexes p50/p50 homodimer and p50/p65 heterodimer are different in the regulation of TNF-α gene promoter (2). The p50 subunit has no activation domain (3, 27); therefore, the p50/p50 homodimer inhibits transcription after binding to the TNF-α gene promoter. The p65 subunit has a transactivation domain (27), and the p50/p65 heterodimer has a much stronger binding affinity to the DNA than the p50/p50 homodimer (3, 27). This leads to the fact that the p50/p65 heterodimer is able to overcome the negative activity of the p50/p50 homodimer and transactivate the gene promoter after it binds to the DNA (27). This mechanism has been demonstrated in the transcriptional regulation of many genes, including IL-2 and interferon-γ (38). The p50/p65 heterodimer was induced by the glass fiber (Fig. 4*A*) and contributed to activation of the TNF-α gene promoter (Figs. 3 and 5).

It may be noted that both clinical and experimental studies (40) indicate that proinflammatory cytokines are important mediators in asbestos-related lung diseases. Several studies (8, 15, 25) have also shown that TNF-α plays an important role in the pathogenesis of pulmonary fibrosis. Although it appears that TNF-α plays an important role in the pathogenesis of lung injury induced by silica and asbestos, there is little information regarding the role of TNF-α in the toxic action of glass fibers. The present data demonstrating

Fig. 5. Inhibition of NF-κB binding activity and TNF-α gene promoter activity by *N*-acetyl-L-cysteine (NAC) and SN50. *A*: suppression of NF-κB binding activity by NAC (20 mM) and SN50 (100 μg/ml) in presence of long fibers. DNA binding activity of NF-κB was determined in gel shift assay. *B*: intensity of NF-κB bands in *A* quantitated with a phosphorimager. NF-κB count was divided by a nonspecific band count that served as a control for protein loading. *C*: inhibition of TNF-α gene promoter by NAC and SN50. Same concentrations of NAC and SN50 were used to treat cells transfected by plasmid reporter that contained TNF-α gene promoter. Long fibers were used to induce reporter activity. Data are means ± SD of reporter activity from 3 independent experiments. *Significant decrease compared with control (17-μm glass fibers alone), $P < 0.001$.

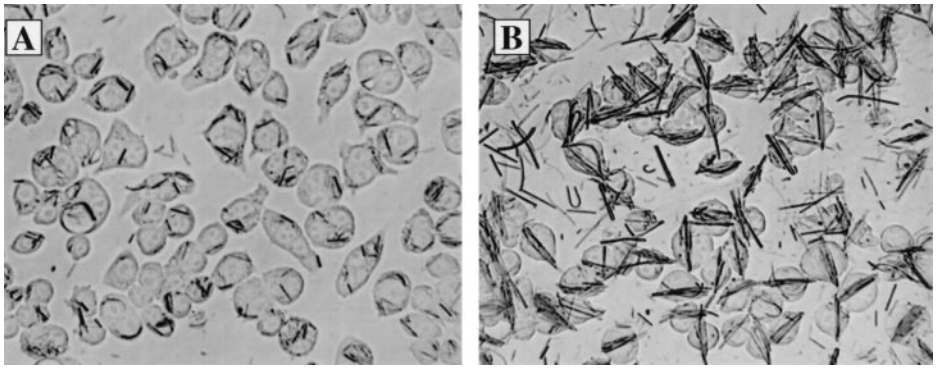


Fig. 7. Morphology of cells exposed to short (*A*) and long (*B*) glass fibers for 16 h.

TNF-α production stimulated by glass fibers suggest that these fibers may exhibit a degree of pathogenic activity by mechanisms similar to silica and asbestos.

The results obtained from the present study show that glass fibers are capable of causing NF-κB activation. Reactive oxygen species may play a key role in NF-κB activation induced by these fibers as demonstrated by the inhibitory effect of NAC. With regard to the generation of reactive oxygen species, it may be noted that the engulfment of foreign substances by macrophages is associated with initiation or enhancement of the respiratory burst in the cell. During the respiratory burst, macrophages and other cellular components generate large quantities of reactive oxygen species. In many cell types, reactive oxygen species have been shown to activate the nuclear translocation of NF-κB by activating reactions, leading to disassociation of an inhibitor (IκB) from NF-κB in the cytoplasm (4, 29). NF-κB protein is found in many different cell types and is a focal point for understanding how extracellular signals induce the expression of specific sets of early-response genes in higher eukaryotes, such as those regulating the secretion of growth promoters (19, 26, 28, 31). It has been suggested that NF-κB activation is crucial to cytoplasmic and/or nuclear signaling when cells are exposed to injury-producing conditions (3). NF-κB serves as a second messenger to induce a series of cellular genes in response to an environmental perturbation. Among the cellular genes regulated by NF-κB are those cytokines regulating inflammation, including TNF-α, IL-6, IL-1, and granulocyte-macrophage colony-stimulating factor (12, 38). NF-κB activates these genes by binding to the NF-κB consensus sequence in their promoters. Through activation of NF-κB, glass fibers may cause expression of many genes, including oncogenes, to initiate inflammation or toxic reactions. Our preliminary study revealed that DNA binding activity of AP-1 was also induced by glass fibers. Because the DNA binding activity of AP-1 is mainly controlled by gene transcription, we expect that c-*jun* and c-*fos* genes are activated by glass fibers.

An important result obtained from the present study is that long fibers act differently from short fibers. The following three major findings should be specially emphasized. First, long fibers are more potent in the stimulation of TNF-α production in macrophages than short fibers. Second, long fibers exhibit a higher potency in causing NF-κB activation than short fibers. Third, macrophage engulfment proceeds differently for long and short fibers. Short fibers were effectively engulfed by macrophages, whereas long fibers were not. This is in agreement with earlier studies (6, 11) that concluded that long fibers are cleared from the deep regions of the lung less effectively than are short fibers. This may be the consequence of failure of macrophages to ingest long fibers completely or their difficulty in moving once they have engulfed a long fiber. The potential of long fibers in the lung to initiate repeated attempts of frustrated phagocytosis may be greater than that of shorter fibers. These frustrated phagocytic events would be expected to generate a greater concentration of reactive oxygen species, cause enhanced NF-κB activation, and stimulate greater production of the inflammatory and fibrogenic cytokine TNF-α. Thus the results obtained from the present study point out that fiber length is a critical factor in determining the toxicity of these asbestos substitutes. It may be noted that, in laboratory studies, fiber mass, but not fiber length, is frequently used in the design and execution of experiments. The present study indicates that fiber length should be taken into consideration in the future study of fiber toxicity and carcinogenicity, as well as in risk assessment activities.

On the basis of the above discussion, it may be postulated that when macrophages attempt to engulf glass fibers, reactive oxygen species are generated because of respiratory burst. Because macrophages cannot engulf the long glass fibers completely, the frustrated cells will generate a higher level of reactive oxygen species. These species, in turn, activate NF-κB. The activated NF-κB binds to the promoter of the TNF-α gene and turns on the transcription to produce TNF-α protein. The generation of reactive oxygen species, activation of NF-κB, and production of TNF-α are involved in the mechanism of toxicity and potential pathogenesis of glass fibers.

In conclusion, *1*) glass fibers are able to stimulate TNF-α production in macrophages. *2*) Glass fibers are also capable of causing NF-κB activation. *3*) Reactive oxygen species are involved in NF-κB activation and in TNF-α production caused by these fibers. *4*) Long fibers are more potent than short fibers for stimulation of

TNF-α production and activation of NF-κB. *5*) TNF-α production induced by fibers is regulated by NF-κB. *6*) Short fibers (7 μm) were effectively engulfed by macrophages, whereas long fibers (17 μm) were not.

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