# CRITICAL ROLE OF FIBER LENGTH IN THE BIOACTIVITY AND CYTOTOXICITY OF GLASS FIBERS

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## Abstract

A critical question in fiber research is the relative contribution of chemical properties vs physical dimensions to the potential pathogenicity of an inhaled fibrous particle. To address this question, it is essential to obtain fiber samples of discrete lengths for investigation. Recently our laboratory has developed a method, which utilized a dielectrophoretic classifier, to separate fiber fractions of narrowly defined lengths. The objective of the present study was to analyze the effects of fiber length on the ability of macrophages to phagocytize these fibers and to determine the potency of fibers of various lengths to activate nuclear transcription and cytokine production and to elicit cytotoxicity. Glass fibers (JM-100) were separated into five discrete size fractions (lengths of 3, 4, 7, 17, and 33 µm). Fibers ≤ 7 µm long were phagocytized by macrophages *in vitro*, while fibers ≥ 17 µm in length were too long to be completely engulfed, resulting in frustrated phagocytosis. There was a clear distinction in the bioactivity and cytotoxicity of fibers too long to be completely engulfed compared to shorter fibers. Glass fiber fractions having 17 µm or 33 µm lengths exhibited similar cytotoxicity on macrophages *in vitro*, measured as lactate dehydrogenase release or inhibition of zymosan-stimulated chemiluminescence. However, these long fibers had a toxic potency nearly two orders of magnitude greater than fiber fractions of 3, 4, and 7 µm lengths. Bioactivity was measured as the ability of glass fiber fractions to activate the DNA binding of the transcription factor, nuclear factor kappa B (NFkB), to activate the gene promoter for tumor necrosis factor alpha (TNFα), and to increase TNFα production by macrophages *in vitro*. Long fibers (17µm) were significantly more potent bioactivators than short fibers (7µm). This bioactivation was inhibited by N-acetyl-L-cysteine, an antioxidant, indicating that the generation of oxidants contributed to this induction. These results suggest that length plays an important role in the potential pathogenicity of fibrous particles with effects being magnified when fibers are too long to be phagocytized completely.

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## Introduction

Asbestos is a term referring to a group of naturally occurring fibrous metallic silicates. Because of their durability and resistance to heat these materials were commonly used in numerous construction and industrial/applications. Use of asbestos is now limited due to evidence linking inhalation of asbestos fibers to pulmonary fibrosis and cancer (Donaldson et al., 1993). Therefore, man-made mineral fibers are now being employed as asbestos substitutes.

It is believe that man-made fibers would be less toxic than asbestos (Bunn et al., 1993). However, a complete understanding of their potential pathogenicity is lacking.

The pathogenicity of fibers is the result of some combination of the physical and chemical properties of the materials. For example, chemical properties would affect fiber durability and the ability to generate reactive species, while length would affect the ability of pulmonary phagocytes to engulf and clear inhaled fibers. Elucidation of the contribution of chemistry vs length to pathogenicity has been hampered by the inability to obtain fiber samples of discrete lengths. Recently, Baron et al. (1994) have described a procedure employing dielectrophoresis to separate fibers according to length. This manuscript describes the use of dielectrophoresis to obtain long and short glass fibers for biological testing of cellular toxicity and the ability to activate the production of inflammatory/fibrotic cytokines, such as tumor necrosis factor alpha (TNFα).

## Methods

### Size-Selected Fiber Fractions

Bulk samples of glass fibers (JM-100) were milled, aerosolized and separated into fiber fractions of discrete lengths by dielectrophoresis (Baron et al., 1994). Fiber samples were collected on Nuclepore filters. Samples were scraped from the filters, weighed, and suspended in sterile water. Samples were then sonicated, diluted and filtered through Nuclepore filters. Filters were sputter coated with gold palladium. Measurement of fiber length, width and counts/mass were determined using a scanning electron microscope (Blake et al., 1998).

### Cells

Functionality and viability studies were conducted using rat alveolar macrophages obtained by bronchoalveolar lavage (Blake et al., 1998). Mouse monocyte cells (RAW 264.7) were purchased from the American Type Culture Collection (Manassas, VA).

### Functionality and Viability Assays

Alveolar macrophages ($2.5 \times 10^6$ cells/ml) were suspended in Eagle's minimum essential medium (pH 7.4) and plated in 96

well microplates. Fibers were heat sterilized, suspended in medium, sonicated, and added to selected wells at various concentrations (fiber counts/ml). Cells were then cultured at 37°C for 18 hr in the absence or presence of glass fibers before fiber toxicity was determined. Decrease in viability was determined as the leakage of lactate dehydrogenase (LDH), a cytosolic enzyme, from damaged cells. LDH in collected supernates was determined using a colorimetric assay based on the reduction of pyruvate (Blake et al., 1998).

Functionality was determined as the ability of cells to generate chemiluminescence in response to zymosan particles (2 mg/ml). Chemiluminescence was enhanced by lucigenin (125 µM) and measured using a microplate luminometer for 1 hr (Blake et al., 1998).

**TNFα, NFkB, and Gene Promoter Assays**

Mouse monocytes (1 x $10^5$ cells/well) were suspended in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine and 100 U/ml penicillin-streptomycin, and added to 96 well microplates. Fibers were heat sterilized, suspended in medium and sonicated prior to addition to selected wells at various concentrations (fibers/cell).

TNFα production was determined by conducting an ELISA assay (Genzyme, Cambridge, MA) on supernates. Nuclear factor kappa B (NFkB) binding to DNA was determined using a gel shift assay (Ye et al., 1999). Briefly, nuclear extracts were prepared. DNA-binding was determined by mixing nuclear extracts (3µg), 1 µg poly (dI-dc), 3µg BSA, 4 x $10^4$ cpm of $^{32}$P labeled oligonucleotide NFkB binding probe, and 12 µl of reaction buffer. The reaction mixture was incubated for 20 min at 22°C and binding measured by gel electrophoresis. NFkB-DNA binding was quantitated by densitometry of the labelled NFkB-oligonucleotide adduct.

TNFα gene promoter activation was determined by transfection of mouse monocytes with a luciferase vector containing a promoter fragment of the human TNF-α gene (Ye et al., 1999). After transfection, the cells were placed in fresh culture medium and exposed to fibers for 16 hr. Gene promoter activation was determined using a light assay for luciferase activity (Promega, Madison, WI).

**Statistics**

Data were compared by two-way analysis of variance (ANOVA). The Student-Newman-Keuls method was used to determine differences among groups. Significance was set at $p \leq 0.05$.

## Results

Glass fiber samples of discrete lengths were obtained by dielectrophoresis. The effectiveness of this procedure is demonstrated in Table 1, which indicates that fiber samples having distinctively different lengths but similar widths were produced. Since the starting material had a uniform composition, the size selected fiber fractions would have identical chemistry.

Exposure of rat alveolar macrophages to glass fibers for 18 hr at 37°C resulted in a dose-dependent decrease in cell functionality (measured as a decrease in zymosan-stimulated chemiluminescence) and a decrease in cell viability (measured as leakage of a cytosolic enzyme from the cells). Long glass fibers (17 µm) were substantially more potent (on an equal fiber number basis) than short fibers (7 µm) as shown in Table 2. Indeed, approximately 1.5-2 orders of magnitude more short fibers were required to exhibit toxicity equivalent to 1 x $10^7$ long fibers/ml.

Exposure of mouse monocytes to glass fibers resulted in a dose-dependent stimulation of TNFα production, with significant stimulation occurring at fiber doses between 5-30 fibers/cell. The time course of stimulation of TNFα production is shown in Table 3. Short glass fibers (7 µm) increased TNFα production by monocytes by 200% and 216% after 6 hr and 16 hr of treatment, respectively. Long fibers (17 µm) were significantly more potent in inducing TNFα release, causing increases of 550% and 960% after 6 hr and 16 hr of treatment, respectively.

Glass fiber-induced TNFα production was associated with activation of the binding of NFkB (a nuclear transcription factor) to DNA and with induction of the activity of the TNFα gene promoter. Long fibers (17µm) were more potent stimulants of these molecular events than short fibers (7µm) as shown in Table 4. Short fibers increased DNA-NFkB binding by 95% and TNFα gene promoter activity by 240% compared to 200% and 575%, respectively, for long fibers.

SN50 is an inhibitor of NFkB. Treatment of monocytes with SN50 (100 µg/ml) not only inhibited fiber-induced NFkB activity but also blocked TNFα gene promoter activation and TNFα production (Table 5). These data indicate a mechanistic link between these events.

Oxidants appear to play a significant role in initiation of molecular events leading to TNFα production. As shown in Table 5, the antioxidant, N-acetyl-L-cysteine (NAC), was a potent inhibitor of NFkB binding, gene promoter activity and TNFα production.

## Discussion

Mechanisms of fiber pathogenicity have included the ability of fibers to damage lung cells and the induction of cytokines and growth factors which induce inflammation, fibrosis and cell proliferation (Donaldson et al., 1993). Data presented in the current study indicate that long glass fibers are

significantly more toxic and induce greater TNFα production than short glass fibers, when exposures are controlled for equal fiber numbers. These data support the Stanton hypothesis that length is a critical factor in pathogenicity (Stanton et al., 1991). The long fibers used in this study had a length of 17 µm, while short fibers were 7 µm long. Blake et al. (1998) and Ye et al. (1999) have shown that 7 µm glass fibers are short enough to be completely engulfed by alveolar macrophages or monocytes, respectively. In contrast, 17 µm fibers are too long to be engulfed and result in frustrated phagocytosis. During frustrated phagocytosis macrophages remain activated and would produce cytokines, which would result in inflammation and cell proliferation. In addition, these activated phagocytes would generate reactive oxygen species, which would lead to cell damage.

TNFα is a pro-inflammatory cytokine linked to the fibrotic process. Data from this study indicate that NFkB is a critical nuclear transcription factor in activation of the TNFα gene promoter and the resultant production of TNFα. Indeed, inhibition of NFkB activation with SN50 causes a 95% inhibition of fiber-induced TNFα production in monocytes. Oxidant species appear to play an important role in NFkB activation, since fiber-induced NFkB-DNA binding, gene promoter activation and TNFα production can be blocked by the antioxidant, NAC. Activation of the respiratory burst during frustrated phagocytosis may represent an oxidant source for this NFkB activation.

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Table 1. Glass Fiber Samples Obtained by Dielectrophoresis

| Sample | Length (µm) | Width (µm) |
|---|---|---|
| 7 µm fibers | 6.5 ± 0.01 | 0.44 ± 0.001 |
| 17 µm fibers | 16.7 ± 0.05 | 0.49 ± 0.001 |

Values are means ± standard errors of 200 fibers evaluated by microscopic analysis.

Table 2. Toxicity of Glass Fiber Samples

| Treatment | Decrease in Functionality | Decrease in Viability |
|---|---|---|
| Control | 0% | 0% |
| 7 µm fibers | 0% | 3% |
| 17 µm fibers | 100% | 55% |

Alveolar macrophages ($2.5 \times 10^5$ cells/ml) were cultured at 37°C for 18 hr in the absence or presence of $1 \times 10^7$ fibers/ml. Functionality was determined as the ability to produce chemiluminescence in response to zymosan (2 mg/ml). Chemiluminescence was enhanced by lucigenin (125 µM) and measured using a microplate luminometer. Decrease in viability was determined as the leakage of lactate dehydrogenase into the supernate. Values are means of 3-4 separate experiments.

Table 3. Time Course of Glass Fiber-induced TNF-α Production by Monocytes

| | TNFα Production | | |
|---|---|---|---|
| Treatment | 3 hr | 6 hr | 16 hr |
| Control | 30 pg | 90 pg | 150 pg |
| 7 µm fibers | 45 pg | 275 pg* | 475 pg* |
| 17 µm fibers | 50 pg | 590 pg ** | 1590 pg ** |

Mouse monocytes ($1 \times 10^5$ cells/well) were cultured at 37°C for various periods of time in the absence or presence of $5 \times 10^5$ fibers. TNFα production was determined by ELISA assay of supernates. Values are means of 3 experiments.

* indicates a significant increase from control

** indicates a significant increase above the effect of short fibers

Table 4. Effect of Fiber Exposure on NFkB Activation and TNFα Gene Promoter Activity

| Treatment | NFkB | TNFα Gene Promoter |
|---|---|---|
| Control | 141 | 1200 |
| 7 µm fibers | 276* | 4100* |
| 17 µm fibers | 423** | 8100* |

Mouse monocytes ($1 \times 10^6$ cells) were exposed to glass fibers at a 5:1 fiber:cell ratio. DNA-NFkB binding was determined by the gel shift assay and bands quantitated by densitometry. TNFα gene promoter activity was determined from cell transfected with a luciferase vector containing the promoter sequence of the TNFα gene.

* indicates a significant increase from control

**indicates a significant increase above the effect of short fibers

Table 5. Inhibition of Events Leading to TNF-α Production by Glass Fiber-Exposed Monocytes

| | Percent Inhibition | | |
|---|---|---|---|
| **Inhibitor** | **NFkB** | **Gene Promoter** | **TNF-α** |
| NAC (20 mM) | 98% | 99% | 97% |
| SN50 (100 µg/ml) | 76% | 88% | 95% |

Mouse monocytes were pretreated for 30 min with an antioxidant (NAC) or an inhibitor of NFkB activation (SN50) and the effect of exposure to long glass fibers (17 µm) determined. Values are means of 3 experiments.