

Magnetometric Evaluation for the Effects of Silicon Carbide Whiskers on Alveolar Macrophages

Mitsuyasu WATANABE^{1*}, Mitsushi OKADA¹, Yoshiharu AIZAWA¹,
Yasuhiro SAKAI², Shohei YAMASHINA² and Makoto KOTANI³

¹Department of Preventive Medicine and Public Health, Kitasato University School of Medicine, Kanagawa 228-8555, Japan

²Department of Anatomy, Kitasato University School of Medicine, Kanagawa 228-8555, Japan

³Department of Electronics, Faculty of Engineering, Tokyo Denki University, Tokyo 101-0054, Japan

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Abstract: Alveolar macrophages are thought to play an important role in fibrogenesis in the lungs caused by various types of exposure to dust. In this experiment, we evaluated the effect of silicon carbide whiskers (SiC) on alveolar macrophages mainly by unique magnetometry and also by established methods such as lactate dehydrogenase (LDH) activity, apoptosis measurement and morphological observations. Alveolar macrophages obtained from Syrian golden hamsters by bronchoalveolar lavages were exposed *in vitro* to Fe₃O₄ for 18 hours as an indicator for magnetometry and SiC for experiments. A rapid decrease of the remanent magnetic field, so called “relaxation”, was observed after cessation of an external magnetic field in macrophages phagocytizing Fe₃O₄ alone, while relaxation was delayed in those concurrently exposed to SiC. Release of LDH from SiC-exposed macrophages into the medium was not significantly higher than the controls, but it increased dose-dependently. Apoptosis was recognized in macrophages exposed to 60 µg/ml of SiC by the DNA ladder detection method and morphological observations. Electron microscopic examination revealed irregular forms of nuclei and organelles in macrophages exposed to SiC. Magnetometry, LDH release and electron microscopic observation indicated mild cytotoxicity of SiC to alveolar macrophages.

Key words: Magnetometry, Silicon carbide whiskers, Alveolar macrophage, Cytoskeleton, Apoptosis

Introduction

There have been many reports on the *in vitro* and *in vivo* toxicity of asbestos in animals¹⁻³. Asbestos fibers are known to cause fibrotic disease of the lung and malignant diseases⁴. Since amosite and crocidolite are legally prohibited for use in many countries including Japan, chrysotile asbestos is the dominant form of asbestos in international commerce today. However, clinical and epidemiologic studies have established that chrysotile causes cancer of the lungs, malignant mesothelioma and asbestosis⁵⁻⁹. Therefore,

nonhazardous substitutes for asbestos are needed to be developed.

There are several man-made-mineral fibers (MMMF) available on the market¹⁰. Silicon carbide whiskers (SiC) are one of MMMF characterized by a monocrystalline whisker form containing 99.6% silicon carbide and 0.16% of Al₂O₃¹¹. Recently SiC has been used as a substitute for asbestos, but the toxicity was evaluated insufficiently. Previously, the cytotoxicity of SiC has been reported by some authors¹²⁻¹⁴. But there have been few reports concerning the toxicity of this material evaluated by measuring the cell functions. Therefore the toxicity should be evaluated in more detail to assess the safety and the application of these fibers.

*To whom correspondence should be addressed.

In this study, we applied magnetometry in evaluating the *in vitro* effects of SiC on macrophages. Cell magnetometry is a modification of pneumomagnetometry conducted first by Cohen¹⁵. Administered iron oxide particles are engulfed by macrophages, and external field magnetizes iron oxide particles located in phagosomes of macrophages. Relaxation, a rapid decrease of the remanent magnetic field strength (RMF) radiated from phagocytized iron oxide particles in macrophages following the cessation of external magnetization was used as an indicator of cellular function. Relaxation is considered to indicate the cytoskeletal function, because relaxation is thought to occur due to random rotation of phagosomes driven by cytoskeleton^{16, 17}.

There have been previous reports observing no effect on the relaxation in limestone-exposed alveolar macrophages¹⁸, and moderate effects by the exposure to chrysotile fibers¹⁹.

Materials and Methods

Reagents

Fe₃O₄ particles with a mean geometric diameter of 0.26 μm (Toda Kogyo Company, Hiroshima) were used as the index of magnetometry. The sample of SiC was provided by Japan Fibrous Materials Research Association. The average short and long geometric diameters of the SiC were 0.3 and 6 μm, respectively (Fig. 1). These samples were suspended in phosphate-buffered saline (PBS) pH 7.4, then stirred by an ultrasonic generator. The whiskers were washed twice by centrifugation and stirred just before use.

Bronchoalveolar lavage

Male Syrian golden hamsters each weighing approximately 100 g were anesthetized by intraperitoneal administration of 100 mg/kg of pentobarbital sodium. Each hamster was bled to death by incision of the abdominal aorta. Bronchoalveolar lavage (BAL) was performed by instilling 3 ml of cold PBS, pH 7.4 containing 0.1% ethylenediaminetetraacetic acid (EDTA) through a tracheal catheter, followed by gentle aspiration. This procedure was repeated nine times. Six hamsters were used for each experiment.

Fluid from all ten lavages was pooled and centrifuged at 1800 rpm for 10 minutes. The cell pellet was resuspended in Eagle's Minimum Essential Medium (MEM, Nissui Pharmaceutical Company, Tokyo) containing 10% fetal bovine serum (FBS). The number of viable cells counted by trypan blue exclusion assay was always over 90%.

Cell culture

The cell suspension was adjusted to a concentration of

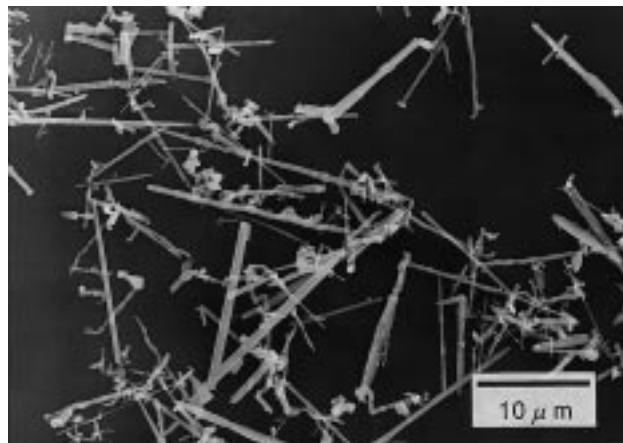


Fig. 1. The TEM image of a silicon carbide whisker.

The average short and long geometric diameters of the SiC were 0.3 and 6 μm.

10⁶/ml and the aliquots of one ml of suspension were seeded into 15 mm culture wells (Nunc Company, Denmark) with a cell disk at the bottom.

As an index of cell magnetometry, 60 μg/ml of Fe₃O₄ particles suspended in PBS were added to all wells. Then, 20, 40, or 60 μg/ml of SiC suspended in PBS was added to each of the experimental group one hour after exposure to Fe₃O₄ particles, while only PBS was added for the controls. The cells were cultured at 37°C in 5% CO₂ for 18 hours. Then, a macrophage-adhered disk was removed from each well to a glass tube containing 1 ml of MEM with 10% FBS.

Magnetometry

Magnetometric measurement was performed by the previously reported method¹⁹. The sample was magnetized by a magnetizer at 70 mT for 10 milliseconds. Immediately after stopping magnetization, RMF was measured for 20 minutes by a fluxgate magnetometer (Magnetoscop, Institut Dr. Foerster, Germany). In order to keep the temperature of air inside the magnetic shield at 37°C, a heater with a thermostat was used.

Thereafter, magnetization field was measured from zero when the magnetization was stopped, because iron oxide particles align completely in response to the external magnetic field and start to misalign as soon as it is removed. A two-minute period of relaxation was added to the exponential function, $B = B_0 e^{-\lambda t}$, where B is the RMF at time t , B_0 the RMF at time = 0, e the exponent, t the time (second) from the termination of magnetization and λ the relaxation rate (decay constant) for 2 minutes²⁰. Linear regression of natural logarithms of RMF at t by the least-squares method was

used to fit the line. B_0 was estimated by extrapolating the function back to time zero.

LDH measurement

One ml of serum-free medium containing 1×10^6 cells was poured into each well with a cell disk at the bottom. Alveolar macrophages exposed to 20, 40 or 60 $\mu\text{g}/\text{ml}$ of SiC were incubated at 37°C in 5% CO_2 for 18 hours. The cell suspension was centrifuged at 1,400 rpm for 10 minutes after incubation. The LDH activity in 50 μl of cell-free supernatant was measured by the Wroblewski-LaDue's method²¹⁾ using the Wako LDH-UV Test (Wako Pure Chemical Industries, Osaka). In order to measure both intra- and extracellular (total) LDH activity, Triton-100 was added to lyse the cells. The LDH release rate (%) was calculated by the following equation; $(\text{LDH from SiC-exposed cells}) \times 100/(\text{total LDH})$.

DNA ladder detecting method

Cultured macrophages exposed to 60 $\mu\text{g}/\text{ml}$ of SiC or PBS for 18 hours were washed with PBS. For DNA extraction, 10 ml of proteinase K (Wako Pure Chemical Industries, Osaka), RNase A (Wako Pure Chemical Industries, Osaka) and 20 ml of 10% SDS (Wako Pure Chemical Industries, Osaka) were added to the samples²²⁾. The samples were incubated at 37°C for 30 minutes. NaI was added to the samples and incubated at 60°C for 15 minutes. After isopropanol precipitation, the DNA was frozen until use.

The sample was resolved in 20 ml of 10 mM Tris-HCl buffer (pH 8.0) with 10 mM EDTA. DNA was electrophoresed on 1% agarose gel, stained briefly with ethidium bromide, and photographed under UV transillumination.

Electron microscopy

Macrophages adhered to a polycationics-treated glass were washed with 0.1 M cacodylate buffer (pH 7.4), and prefixed with 1% glutaraldehyde at 4°C for 3 hours. After being washed, they were postfixed with 1% OsO_4 at 4°C for 3 hours, and washed with 0.1 M cacodylate buffer. For transmission electron microscopic (TEM) observation, the cells were examined with a HITACHI H-600 after dehydration, resin embedding, ultra-thin sectioning and electron staining. For observation under a scanning electron microscope (SEM), the cells were examined with a HITACHI S-4500 FE after ion sputter coating by platinum.

Statistical analysis

Results are expressed as means \pm S.E. from six animals

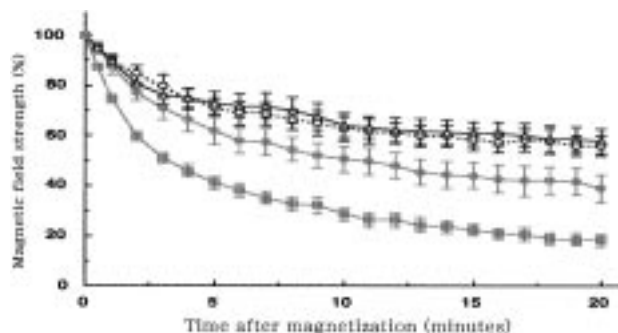


Fig. 2. Relaxation curve.

Relaxation curve in the alveolar macrophages exposed in vitro to various doses of silicon carbide whiskers and PBS as controls. The normalized percent means of RMF with S.E. from 6 hamsters were plotted at the initial RMF rate of as 100%. Significantly delayed relaxation curves were demonstrated in the groups exposed to 20 (— —), 40 (--- ---) and 60 $\mu\text{g}/\text{ml}$ (— ·—) compared with that of the PBS-treated group used as controls (— ·—).

of each group. Statistical differences among group averages were determined using one way or two-way analysis of variance and Scheffe's method.

Results

Magnetometry

The averages of RMF obtained from macrophages exposed to SiC and PBS are plotted in Fig. 2. Relaxation was delayed in the groups exposed to SiC compared with the controls. Statistically significant differences were found among the controls compared with the groups exposed to 20, 40 and 60 $\mu\text{g}/\text{ml}$ of SiC by two-way analysis of variance ($p < 0.001$). As a result of multiple comparisons by Scheffe's method, significant differences at 5% level were recognized between the controls and the 20 $\mu\text{g}/\text{ml}$ of SiC-exposed groups, 0.1% level were recognized between the controls and the 40 $\mu\text{g}/\text{ml}$ of SiC-exposed groups and also between the controls and the 60 $\mu\text{g}/\text{ml}$ of SiC-exposed groups.

The decay constants of the SiC-exposed groups showed significantly lower values than those of the control group by multiple comparison conducted by Scheffe's method (Table 1).

LDH release

The LDH release from the cytoplasm of the pulmonary alveolar macrophages has been used as an index of sublethal cytotoxicity. The mean LDH release rates from the macrophages exposed to various concentrations of SiC were low, but increased dose-dependently. No statistically

Table 1. Decay constants and LDH release rates

	Controls	Silicon carbide whisker ($\mu\text{g/ml}$)		
		20	40	60
Decay constant ($\times 10^{-3} \cdot \text{sec}^{-1}$)	4.49 \pm 0.32	2.26 \pm 0.46***	1.59 \pm 0.33***	1.87 \pm 0.40***
LDH release rate (%)	3.66 \pm 0.51	4.27 \pm 0.64	6.09 \pm 1.21	8.77 \pm 1.91

Values are means \pm S.E. from 6 hamsters in each experiment. *** $p < 0.001$ compared with the PBS-added control cells. No significant difference was found in LDH release rates.

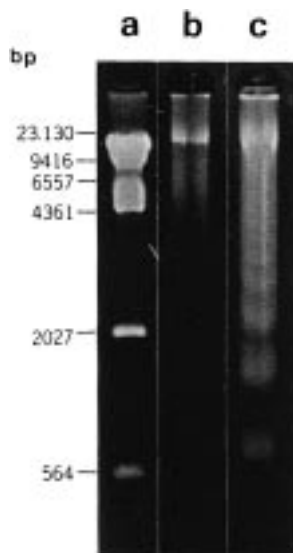


Fig. 3. Electrophoretic analysis of DNA extracted from alveolar macrophages.

Macrophages exposed to 60 $\mu\text{g/ml}$ of silicon carbide whisker for 18 hours (Lane c), PBS-added controls (Lane b). Indicators with various kilobase pairs (kbp) are shown in Lane a.

significant differences were found in these groups compared with the control group (Table 1).

Apoptosis

DNA fragmentation was found in the samples of SiC-exposed macrophages by the DNA ladder detection method (Fig. 3).

Electron microscopy

Macrophages treated with PBS appeared intact (Figs. 4a), while some of those exposed to SiC were penetrated with fibers (Fig. 4b) in the SEM images. The obvious destruction of cell membranes was not recognized.

In the TEM images, central location of vacuoles and intracellular organelles and the concentration of chromatin in the nucleus were observed in some SiC-exposed

macrophages (Fig. 4d) in contrast with the control (Fig. 4c). These findings are consistent with apoptotic changes. Apoptotic changes were observed in about 10% of cells exposed to SiC compared with 2% of the normal control in the TEM images. Remarkable changes of the cytoskeleton could not be found in these images.

Discussion

It has been reported that asbestos causes respiratory disorders such as asbestosis, pleural changes, lung cancer and mesothelioma in humans⁵⁻⁹. SiC and other substitutes for asbestos are used in the workplaces for that reason.

However, the toxicity of SiC has not been evaluated sufficiently. Recently, some authors reported toxicity of SiC¹²⁻¹⁴. In *in vitro* experiments, toxicity was evaluated by the production of tumor necrosis factor (TNF)¹² and the inhibition of the cloning efficiency of cell lines¹³. In *in vivo* experiments, Vaughan *et al.*¹⁴ observed histological changes such as multiple pulmonary granulomas with occasionally occluded airways induced by chronic exposure to SiC by transtracheal instillation.

In rabbits, intratracheally instilled gallium arsenide caused a delay in relaxation and it also caused diffuse chemical pneumonitis²³. A delayed relaxation was also induced by cigarette smoking in the human model²⁴. On the other hand, relaxation was accelerated when rats were infected with the influenza virus²⁵. Brain *et al.*²⁰ observed that the iron particles were retained in the macrophages for several months and associated with relaxation.

In *in vitro* experiments, there have been previous reports observing no effect of relaxation in alveolar macrophages by exposure to limestone¹⁸, and mild effects by silica²⁶ and chrysotile fibers¹⁹. In the present study, *in vitro* exposure of 20 $\mu\text{g/ml}$ or higher doses of SiC caused a delay in relaxation on the bases of relaxation curves and decay constants. Decay constants indicate the speed of decreasing RMF for the first 2 minutes.

Cytoskeleton including microtubules, microfilaments, and

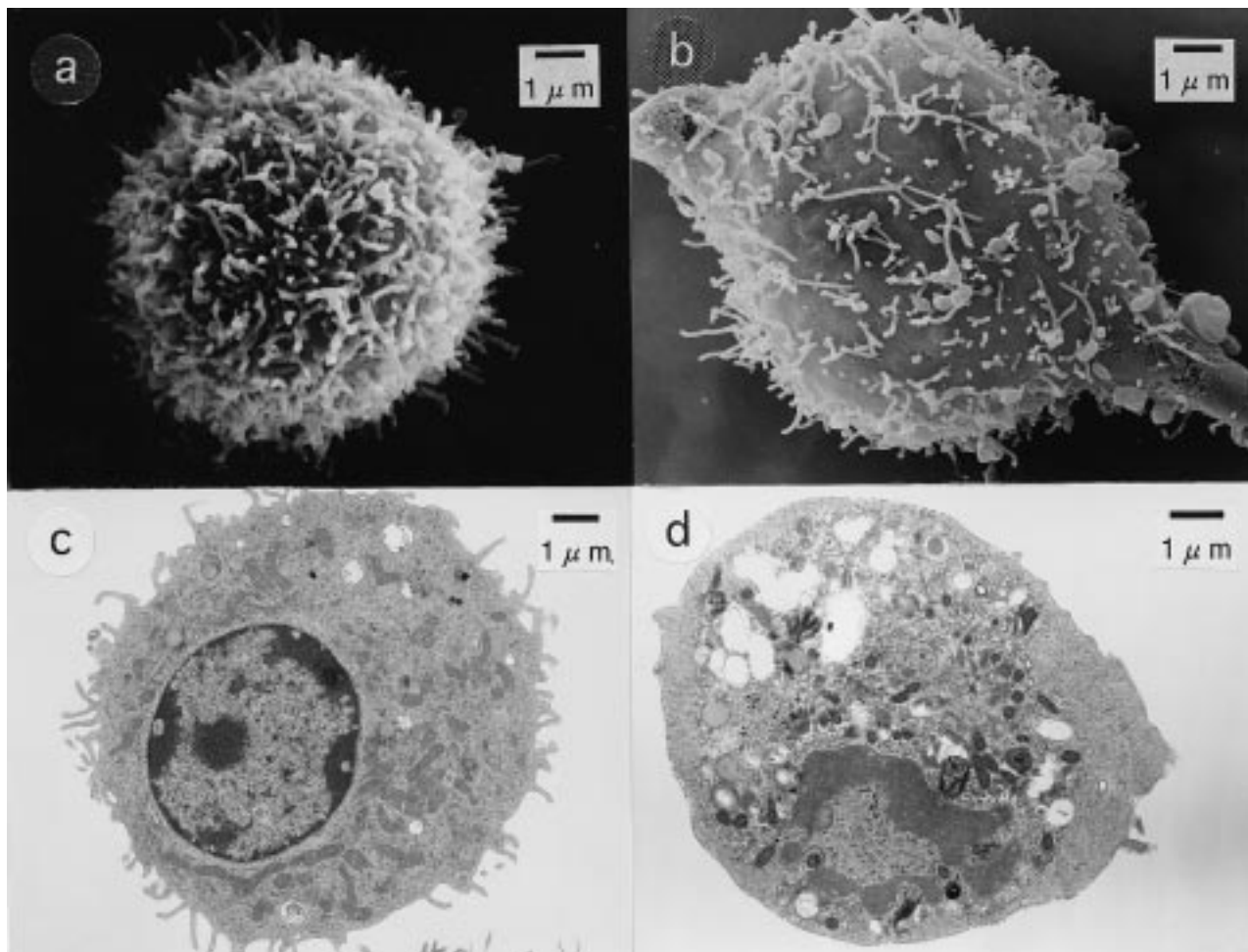


Fig. 4. Representative electron microscopic images.

(a) In this SEM image, the surface of the cells possessed a number of filopodia. (b) One fiber of a silicon carbide piece went straight to a macrophage exposed to a silicon carbide whisker. (c) In this TEM image, the shape of the macrophage appeared round. A considerable number of filopodia, mitochondria and phagosomes, can be observed in the cell. (d) The cells exposed to silicon carbide whisker demonstrated diminished filopodia and irregular forms of nuclei with condensed chromatin. The vacuolar formation and mitochondria had a tendency to create dense structures, and the central location of intracellular organelles can be observed.

intermediate filaments has been known to play important roles in the integrity of cytoplasm, transport of organelles, cell polarity and the mobility of cells²⁷⁻³⁰. The mechanism of impaired cytoskeletal function due to SiC has not been elucidated. Relaxation is thought to occur due to the random rotation of phagosomes containing magnetized iron oxide particles in the intracellular environment^{15, 17, 20}. Phagosomes containing extracellular substances try to digest them and move inside the cells. The polymerization and depolymerization of cytoskeleton, mainly microtubules, play an important role in the movement of phagosomes. Erickson *et al.*³¹) observed that the polymerization of cytoskeleton was associated with guanosine triphosphate hydrolysis.

Nitric oxide inhibits irreversibly the enzymes for ATP

production by mitochondrial respiration^{32, 33}. Leanderson³⁴) suggested that nitric oxide is present on the surface of SiC. Insufficient ATP production due to nitric oxide from SiC may cause impaired movement and polymerization of the cytoskeleton. This suggests that the impaired cytoskeleton function is attributed to the altered energy metabolism. The rotation of phagosomes assessed by relaxation is thought to indicate cytoskeletal function. Since ultramicroscopic observations could not reveal conclusive morphological changes of the cytoskeleton, relaxation might be only a sensitive indicator of the response to exposure by cytoskeleton.

Koshi *et al.*¹) observed that an appreciable amount of LDH was released from cells exposed to asbestos. The release of

LDH in the extracellular fluid in this study was not significantly high and as low as those exposed to chrysotile fibers in another study¹⁹). However, it is difficult to compare the present study with the previous one, because serum-free medium was not used in the previous study. In this study, in spite of low LDH release rates, they increased dose-dependently. Therefore, it is possible that the sublethal cytotoxicity of SiC may be demonstrated by a higher dose of SiC.

As for apoptosis, DNA fragmentation being specific for apoptosis was observed in the SiC-exposed macrophages by the DNA ladder detection method. A previously study showed an induction of apoptosis due to chrysotile fiber exposure and suggested a relationship between apoptosis and fibrosis³⁵). Asbestos-induced apoptosis appeared to involve one or more members of the interleukin-converting enzymes which preceded endonuclease activation³⁵). In this study, the DNA ladder was observed and electron microscopic examination revealed a cellular appearance consistent with apoptosis in some portion of macrophages exposed to SiC. Albina *et al.*³⁶) suggested that nitric oxide caused apoptosis in the cells due to the suppression of the electron transport chain.

For the assessment of the cytotoxicity of SiC, it is important to evaluate the functional, biochemical and morphological aspects. Magnetometry is useful for the evaluation of cell function. In the LDH release assay, obvious evidence of toxicity was not detected. On the other hand, a significant difference was found in magnetometric observation between SiC and the control groups. Therefore magnetometric observation was a more sensitive method in evaluating the cytotoxicity of SiC than others.

Magnetometry appears useful for screening the *in vitro* toxicity of SiC. However, the systemic effect of SiC should be confirmed by *in vivo* experiments.

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