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Original Article

The Cytotoxicity of Microglass Fibers on Alveolar Macrophages of Fischer 344 Rats Evaluated by Cell Magnetometry, Cytochemisry and Morphology

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Abstract

Objectives: The toxicity of microglass fibers (MG), one of the man-made mineral fibers, has not been sufficiently evaluated. The aim of the current study was to evaluate the cytotoxicity of MG *in vitro*.

Methods: Alveolar macrophages were obtained from the bronchoalveolar lavage of male F344/N rats. The macrophages were exposed to MG at concentrations of 0, 40, 80, 160 and 320 μg/ml. The effects of MG on the macrophages were examined by cell magnetometry, LDH assay and morphological observation.

Results: In the cell magnetometry experiment, a significant delay of relaxation (the reduction of remanent magnetic field strength) was observed in the cells treated with 160 and 320 μg/ml of MG in a dose-dependent manner. A significant increase in LDH release was also observed in the cells with 160 and 320 μg/ml in a dose-dependent manner. Changes in the cytoskeleton were observed after exposure to MG, by immunofluorescent microscopy using an α-tubulin antibody.

Conclusions: The cytotoxicity of MG on alveolar macrophages was demonstrated with cell magnetometry. The mechanism of the toxic effects of MG was related to cytoskeleton damage.

Key words: magnetometry, alveolar macrophage, microglass fibers, man-made mineral fibers

Introduction

The toxicity of asbestos has been reported in an *in vitro* study (1). The exposure to asbestos is also related to several diseases, such as mesothelioma and lung cancer (2). As fibers safer than asbestos, man-made mineral fibers (MMMFs) have been developed, and are used widely as new constructional and industrial materials (3). MMMFs are considered safer than asbestos, based on their physical and chemical characteristics (4, 5), which are related to the toxicity of the fibers. However, Stanton et al. (6) have suggested that not only asbestos, but also any long and thin fibers which are retained in the body for long periods of time, could cause lung injury, fibrosis and lung cancer. Actually, the safety of MMMFs either long or short in length, has not been fully confirmed (7).

Microglass fibers (MG) are one MMMF, with short and extremely small fiber diameters. It has been used as a filter for microparticles in the air and water, an insulating material in batteries, and insulation and sound-proofing in airplanes and the space industry since the 1930's (3). Due to the shorter history of MG use compared to other MMMFs, there are few reports of epidemiological and experimental studies for MG. Thus the safety of MG is required to be evaluated.

Alveolar macrophages play an important role in the defense mechanism in the lung (8). Since fibers are taken into the body by inhalation, the evaluation of the toxicity on alveolar macrophages will provide useful information for the safety of MG. Cell magnetometry (9) is used for screening the toxic effects of new materials on alveolar macrophages. In this study,

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the cytotoxicity of MG on alveolar macrophages was examined by cell magnetometry. In addition, LDH measurement, morphological examinations by immunofluorescent microscopy and electron microscopy were used for the evaluation of the toxicity of MG.

Materials and Methods

Materials

Microglass fibers (MG) were supplied by the Japan Fibrous Materials Research Association (Tokyo). Particles of Fe_3O_4 (Toda Kogyo Co., Hiroshima) were used as an indicator material in the cell magnetometry. Chrysotile fibers (CF) as the positive control of cytotoxicity, were supplied by the Japan Association for Working Environment Measurement (Tokyo).

The geometrical mean of the length and width and the geometrical standard deviation of MG were 3.00±2.22 and 0.24±2.35 μm, respectively (Fig. 1a). The diameter of Fe_3O_4 ranged from 0.08 to 0.57, and the geometrical mean diameter of Fe_3O_4 was 0.26 μm. The geometrical mean of the length and width and the geometrical standard deviation of CF were 1.74±1.17 and 0.056±0.035 μm, respectively (Fig. 1b). The composition of MG used in the present study was Si 60.496%, Ca 30.545%, K 6.934%, Al 1.509%, Fe 0.414% and Zn 0.102%. The composition of CF was Mg 54.11%, Si 40.85%, and Fe 5.04%. These materials were suspended in phosphate buffered saline (PBS), pH 7.4, sterilized by autoclaving (1.1 atmospheric pressure, 121°C, 20 minutes), and mixed well before use.

Harvest and preparation of alveolar macrophages

Male F344/N scl rats (CLEA Japan Inc., Tokyo), with each rat weighing approximately 200–250 g, were anesthetized by an intraperitoneal injection of pentobarbital (100 mg/kg). The rats were euthanized by cutting the abdominal aorta through a midline abdominal incision. The trachea was exposed, and a silicon tube (Atom Intravenous Catheter 5 French for cut-Down, Atom Medical Co., Tokyo) was inserted and fixed in the trachea. While massaging the chest area, 4 ml cold PBS including 0.1% EDTA (pH 7.4), which was sterilized by filtration through a Millipore filter, was injected repeatedly through the tube into the lungs, and the bronchial alveolar lavage (BAL) was collected. After repeating the injection 10 times, alveolar macrophages were collected from the BAL by centrifugation at 1800 rpm for 10 minutes. The cell pellets were suspended in serum-free medium (Macrophage-SFM liquid, Life Technologies, Inc., Rockville, MD, USA), and the number of cells was counted with a hematocytometer. The viability of cells was checked using the trypan-blue exclusion test, confirming that most cells were viable. Morphological observation of the cells was performed simultaneously, and most of the cells showed similar images. Approximately 4.0–6.0×10^6 cells per animal were obtained. It was reported the cells collected from the BAL were almost all alveolar macrophages (10).

The care and treatment of rats were in accordance with the guidelines established by the Institutional Animal Care of Kitasato University School of Medicine and were approved by the Use Committee.

Exposure and cell culture

The alveolar macrophages, prepared as described above, were plated on a 1-cm cell disc in the bottom of a well at 1×10^6 cells/well in a 4-well cell culture plate (Nunc A/S, Roskilde, Denmark).

As an index of cell magnetometry, 50 μg/ml Fe_3O_4 suspended in PBS was added to each well. Sixty minutes after the exposure to Fe_3O_4, MG suspended in PBS was added to the well at concentrations of 40, 80, 160 and 320 μg/ml in each experimental group. Fifty μl of PBS was added in the negative control group. As the positive control, CF suspended in PBS was added in a well at a final concentration of 40 μg/ml. The plates were incubated in a humidified incubator at 37°C with 5% CO_2 overnight (18 hours).

Cell magnetometry

The cell magnetometry was performed as previously

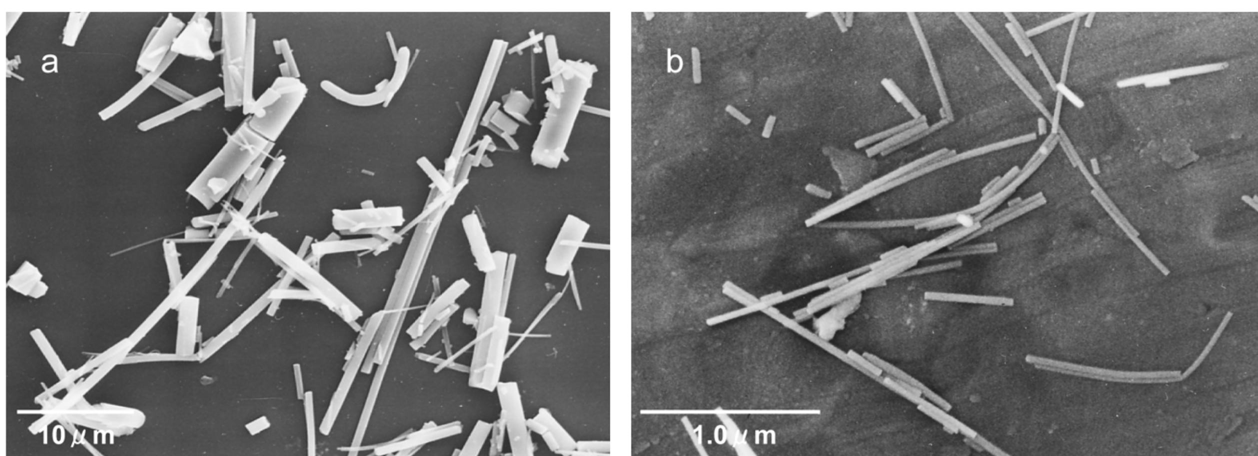

Fig. 1 Scanning electron micrographs. (a) Micrographs of MG fibers. The white bar represents 10 μm in length. The geometrical mean of the length and width and the geometrical standard deviation of MG were 3.00±2.22 and 0.24±2.35 μm. (b) Micrographs of CF fibers. The white bar represents 1.0 μm in length. The geometrical mean of the length and width and the geometrical standard deviation of CF were 1.74±1.17 and 0.056±0.035 μm.

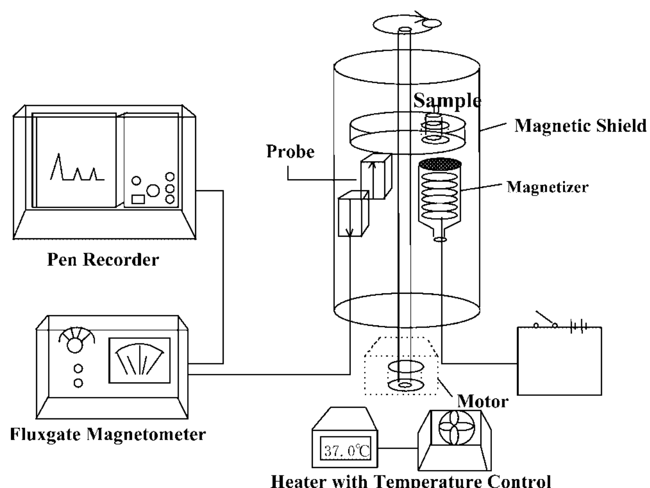

Fig. 2 The cell magnetometry apparatus.

reported by Keira et al. (9). Briefly, alveolar macrophages adhered to the cell disc were removed and transferred to a glass tube containing 1 ml serum-free medium. These cells were magnetized at 70 mT for 1/100 second using the magnetizer of a cell magnetometry apparatus (Fig. 2). Immediately after the magnetization, the remanent magnetic field strength (RMF) was measured for 20 minutes with a Fluxgate magnetometer (Institut Dr. Foerster, Reutlingen, Germany) and recorded with a pen recorder. The sample plate passed over the probe once every 6 seconds. The temperature was maintained at 37°C by a thermostated air fan under a magnetic shield.

The RMF over 20 minutes after magnetization was plotted. The rapid reduction of RMF after the termination of magnetization is called relaxation. The relaxation at 20 minutes after magnetization, B_{20} (%), was obtained from the formula $B_{20}\times 100/B_0$, where B_{20} is the RMF 20 minutes after termination of magnetization. The logarithm of the RMF for the first 2 minutes after magnetization were calculated to obtain the intercept with the y-axis. The decay constant (λ) was obtained from the formula $B=B_0e^{-\lambda t}$, where B_0 is the intercept with the y-axis, B is the RMF at t seconds after the termination of external magnetization.

LDH measurements

The release of lactate dehydrogenase (LDH) from alveolar macrophages was used as the degree of cellular membrane damage. For LDH measurements, the alveolar macrophages were incubated as described above. For measuring the total (both intracellular and extracellular) LDH activity, 0.2% TritonX-100 was added to the additional PBS cultured cells.

After centrifugation at 1400 rpm for 5 minutes, the LDH activity in the supernatant was measured by the Wroblewski-LaDue method (11) using a LDH-UV test kit (Wako Pure Chemical Industries, Ltd., Tokyo). The LDH activity was estimated from the decrease in absorbance for 2 minutes using an U-3210 type Hitachi automated spectrophotometer (Hitachi Ltd., Tokyo). The total LDH activity was determined from the supernatant of TritonX-100 added samples. The LDH release rate (%) was calculated from the following equation: (LDH activity in the supernatant of the cells)×100/(total LDH activity).

Morphological observation

The macrophages, adhered to poly-L-lysin coated glass disc in a glass tube were exposed to 160 μg/ml MG, and incubated at 37°C in 5% CO_2 overnight (18 hours). The cultured cells were fixed with acetone for 10 minutes. After washing and blocking, cells were stained for 90 minutes with α-tubulin FITC conjugate (Sigma-Aldrich, Japan, Tokyo) by the direct antibody method, dehydrated with 50% ethanol, and mounted in glycerol. The cells were examined by immunofluorescent micrography (Axioplan 2., Zeiss Co., Oberkochen, Germany).

For electron microscopic observations, macrophages exposed to 160 μg/ml MG or 50 μg/ml CF were incubated at 37°C in 5% CO_2 overnight (18 hours) in a plastic tube. After the incubation, the cells were adhered to a polycationics-treated glass slide by pipetting. The adhered cells were washed with 0.1 M cacodylate buffer (pH 7.4), and prefixed with 1% glutaraldehyde at 4°C for 3 hours. The cells were washed again with 0.1 M cacodylate buffer (pH 7.4), and they were postfixed with 1% osmium tetroxide at 4°C for 3 hours, and finally washed with 0.1 M cacodylate buffer (pH 7.4). For the observation with the transmission electron microscope (TEM), the fixed cells underwent the process of dehydration, resin embedding, ultrathin sectioning with an ultramicrotome and electron staining with uranyl acetate and lead citrate. TEM observation of the cell was performed using a Hitachi H-600 (Hitachi Ltd., Tokyo). For the observation with the scanning electron microscope (SEM), the cells underwent the process of conductive staining, dehydration, drying and conductive treatment. SEM observation of the cell was performed using a Hitachi S-4500FE (Hitachi Ltd., Tokyo).

Statistical analysis

The data are shown as the mean±standard error of the results obtained from 6 rats for both control and experimental groups. Statistical differences among the groups were examined by one-way analysis of variance in StatView 4.02 (Abacus Concept, Berkeley, CA), and the multiple comparison test (the significant level $p<0.05$) was performed by the Scheffe's method.

Results

Cell magnetometry

The relaxation curve of the RMF during the 20 minutes after the magnetization by cell magnetometry for each group is illustrated in Fig. 3. The mean values of B_{20} (%) of the 160 and 320 μg/ml group were significantly lower than that of the PBS group. In the 40 μg/ml CF group, B_{20} (%) was significantly lower than that of the PBS group. In addition, a significant difference in the mean value of B_{20} (%) was observed between the 40 μg/ml MG group and 40 μg/ml CF group.

The decay constant for the two minutes after magnetization is demonstrated in Fig. 4. A significant difference was observed between the PBS group and the 320 μg/ml MG group. The decay constant became smaller as the MG concentration

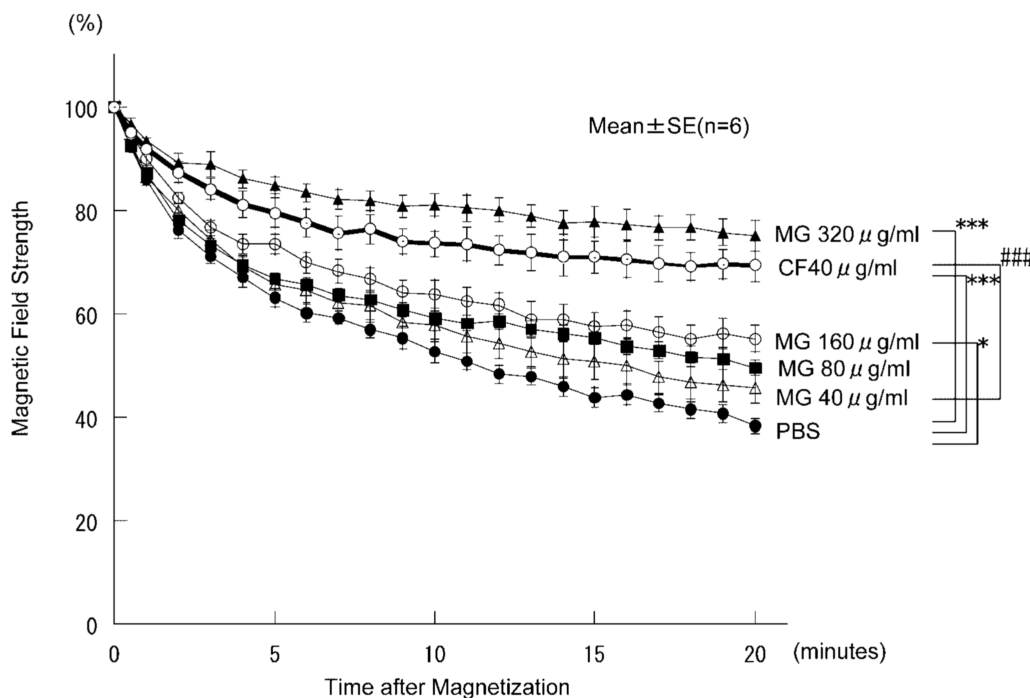

Fig. 3 The relaxation curves of alveolar macrophages exposed to MG or CF after magnetization. The alveolar macrophages plated at 1×10^6 in each well with a 1-cm cell disc mounted in the bottom of a 4-well cell culture plate. Fifty μg/ml Fe_3O_4 (as an index of cell magnetometry) suspended in PBS was added to each well. After 60 minutes, MG was added at concentrations of 40, 80, 160 and 320 μg/ml and CF was added at the concentration of 40 μg/ml (n=6 for each group). The cells were incubated overnight (18 hours) at 37°C. Alveolar macrophages adhering to the cell disc were removed and transferred to a glass tube containing 1 ml serum-free medium. The cells were magnetized at 70 mT for 1/100 second using the magnetizer of the cell magnetometry apparatus. The remanent magnetic field strength (RMF) was expressed as a % of the initial value of RMF. The mean of RMF over 20 minutes after the magnetization was plotted. Each bars experiment standard errors in each group. The mean values of the B_{20} (%) which were compared among the group, * $p<0.05$, *** $p<0.001$ (comparison among MG group), ### $p<0.001$ (comparison to the CF group).

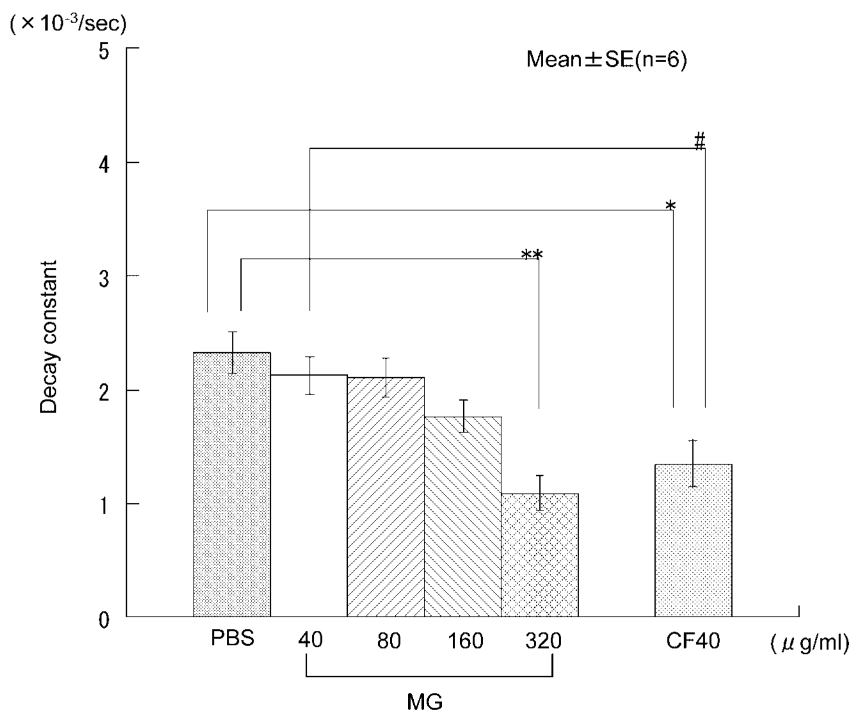

Fig. 4 The effects of MG or CF on decay constant of magnetized alveolar macrophages. The condition of the incubation and magnetization of alveolar macrophages were the same as described in Fig. 3. The logarithm of the RMF for the first 2 minutes after the magnetization were calculated to obtain the intercept with the y-axis. The decay constant (λ) for the first 2 minutes after magnetization was obtained from the formula $B=B_0e^{-\lambda t}$, where B_0 is the intercept with the y-axis, B is the RMF at t seconds after the termination of external magnetization. Each bar shows experiment mean values, and error bars represent standard errors (n=6). The mean values of the decay constant (λ) which were compared among the groups, * $p<0.05$, ** $p<0.01$ (comparison among MG group), # $p<0.05$ (comparison to the CF group).

increased. The decay constant in the 40 μg/ml CF group was significantly lower than that in the PBS group. In addition, there was a significant difference between the 40 μg/ml MG and 40 μg/ml CF groups.

LDH measurement

The LDH activity of the supernatant of each group is illustrated in Fig. 5. The exposure to MG increased LDH release in a dose-dependent manner. The mean values of 160 and 320 μg/ml group were significantly lower than that in the PBS group. There was a significant difference between the 40 μg/ml MG and 40 μg/ml CF groups.

Morphological observations

Immunofluorescent micrographs of macrophage with FITC anti-α tubulin are demonstrated in Fig. 6. Fig. 6a shows the fine microtubule network of macrophages in the PBS group. Fig. 6b shows the microtubule network of macrophages exposed to 160 μg/ml MG. The MG fiber protruded from the cytoplasm because it was too large to be engulfed by a single macrophage. The microtubule of macrophages exposed to MG appeared to be aggregated compared with the PBS group. The microtubule network of macrophages exposed to 40 μg/ml CF is shown in Fig. 6c. The aggregation of microtubules was marked in the macrophages engulfing CF among the cells in this experiment.

An electron micrograph of a normal macrophage by SEM is illustrated in Fig. 7a. A large number of microvilli on the surface of a ball-shaped cell were observed. As shown in the SEM image of macrophages engulfing MG (Fig. 7b), MG fibers were too long for the macrophage to engulf it completely. Under the incomplete phagocytosis, the microvilli, which protruded in the direction of the fibers, did not surround the fibers. The cytoplasmic membrane was stretched in the direction of the fibers. Fig. 7c is a SEM image of macrophages engulfing CF. Very thin CF fibers were tangled around macrophages, with the depletion of microvilli from the surface of macrophages. A TEM image of a normal macrophage is shown in Fig. 8a, and that of macrophage engulfing MG is shown in Fig. 8b. In the MG-exposed macrophage, phagocytized MG fibers were observed in the cytoplasm, with vacuoles around them. Fig. 8c shows a TEM image of macrophage engulfing a CF. The macrophage with thin phagocytized CF fibers has many vacuoles in the cytoplasm as well as nuclear condensation.

Discussion

MG, one of the narrow MMMFs, is frequently used as a highly efficient insulation material for airplanes and in the space industry. The effects of MMMFs on humans and experimental

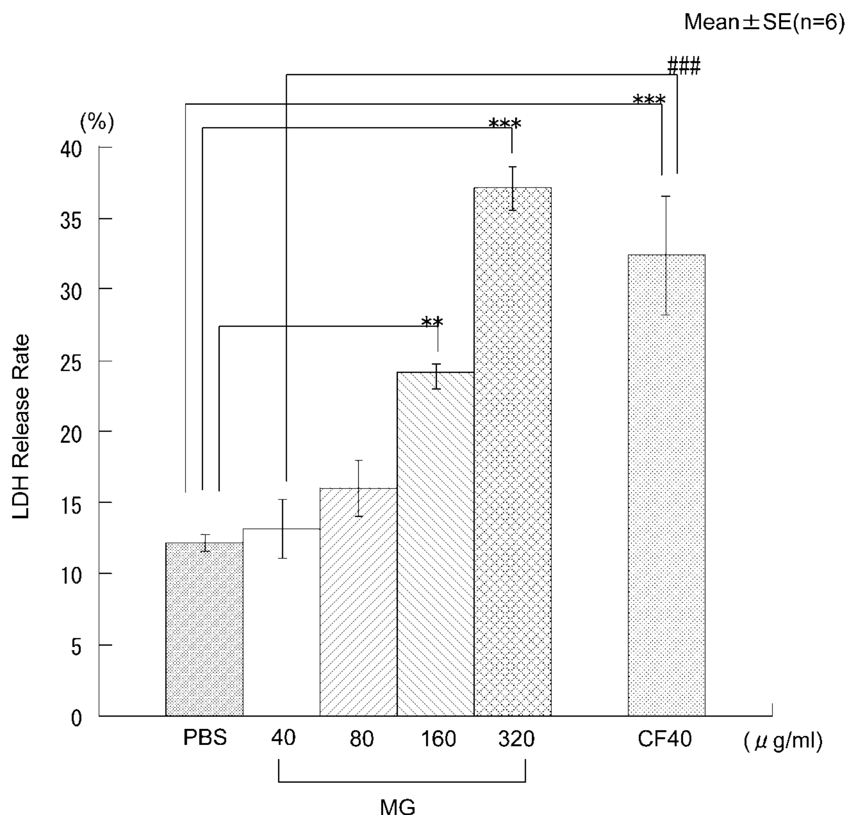

Fig. 5 The effects of MG or CF on LDH release from alveolar macrophages. The alveolar macrophages were incubated overnight (18 hours) in 5% CO_2 incubator at 37°C. For measuring the total (both intracellular and extracellular) LDH activity, 0.2% TritonX-100 was added to the additional PBS cultured cells. The LDH activity in the supernatant was measured by the Wroblewski-LaDue method using a LDH-UV test kit. The LDH release rate (%) was calculated from following equation: (LDH activity in the supernatant of the cells)×100/(total LDH activity). Each bar shows experiment mean values, and error bars represent standard errors (n=6). The mean values of LDH release which were compared among the group, ** p<0.001, *** p<0.001 (comparison among MG group), ### p<0.001 (comparison to the CF group).

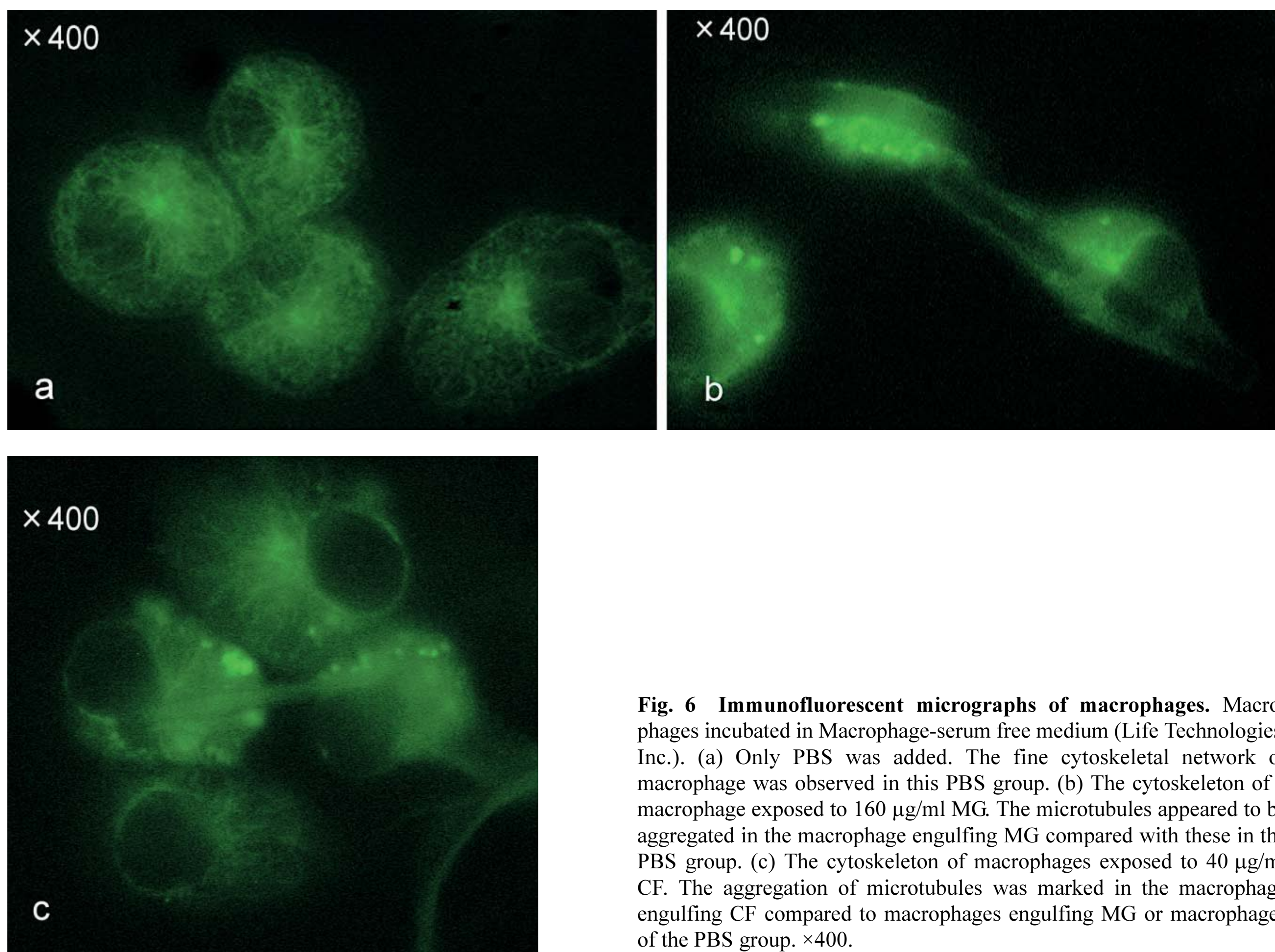

Fig. 6 Immunofluorescent micrographs of macrophages. Macrophages incubated in Macrophage-serum free medium (Life Technologies, Inc.). (a) Only PBS was added. The fine cytoskeletal network of macrophage was observed in this PBS group. (b) The cytoskeleton of a macrophage exposed to 160 μg/ml MG. The microtubules appeared to be aggregated in the macrophage engulfing MG compared with these in the PBS group. (c) The cytoskeleton of macrophages exposed to 40 μg/ml CF. The aggregation of microtubules was marked in the macrophage engulfing CF compared to macrophages engulfing MG or macrophages of the PBS group. ×400.

animals have been evaluated by the International Agency for Research on Cancer (12). MG was classified into the category 2B, which has sufficient carcinogenic properties in experimental animals, but is only possibly carcinogenic to humans, due to insufficient evidence. Beck et al. (13) reported that alveolar macrophages exposed to glass fibers in which the width is thicker than MG caused cytotoxicity. The cytotoxicity of glass fibers was similar to that of CF. Therefore, similar cytotoxicity can be expected with MG. We evaluated the cytotoxicity of MG on alveolar macrophages, which has a role of the primary defense in the lung.

Traditionally, cytotoxicity has been examined by the leakage of LDH activities from the cells and morphological examinations. In addition to these traditional methods, we applied magnetometry, which was originally described by Cohen et al. (14). The toxicities of the following chemical substances have been investigated by *in vitro* cell magnetometry using alveolar macrophages of rodents: limestone (15), chrysotile (9), gallium arsenide (16), silicon carbide whisker (17), titanium oxide (18), cadmium chloride (19), arsenic chloride (20), photocopier toner (21), rock wool (22) and cadmium oxide (23).

In these methods, magnetic particles (iron oxide particles) are engulfed by rat alveolar macrophages obtained by BAL. The cells are then magnetized externally. The RMF, which is generated from magnetized iron oxide particles in cells, is measured right after external magnetization. The rapid reduction of RMF that occurs upon external magnetization is called relaxation. The magnetic moments of the particles engulfed by cells become unidirectional due to the external magnetization. After the termination, the magnetic unidirection is lost with time due to the random rotation of phagosomes in the cells. Brain et al. (24) demonstrated the decrease in RMF over time is not due to leakage of iron oxide particles from the cells, but from the rotation of iron oxide by the movement of phagosomes, since the iron oxide particles remained in the cell after the relaxation occurred. It is considered that polymerization and depolymerization of the cytoskeleton, which is composed of microtubules, microfilaments and intermediate filaments play important roles in the movement of phagosomes (25, 26). If the cytoskeleton is damaged physically and chemically by the addition of hazardous substances, the delay of relaxation would occur and be detected by cell magnetometry (24, 27).

In the present study, the B_{20} (%) of the 160 and 320 μg/ml MG groups was significantly higher than that of the PBS group, which demonstrates a delay in relaxation. The decay constant, λ was also significantly lower than the PBS, which means the delay of the relaxation occurred at an early stage after external magnetization. In the morphological observation with immunofluorescent staining, changes in the cytoskeleton were demonstrated in macrophages that engulfed the fibers. The mild morphological observation of MG-exposed macrophages by TEM suggested the possible involvement of the cytoskeleton. It

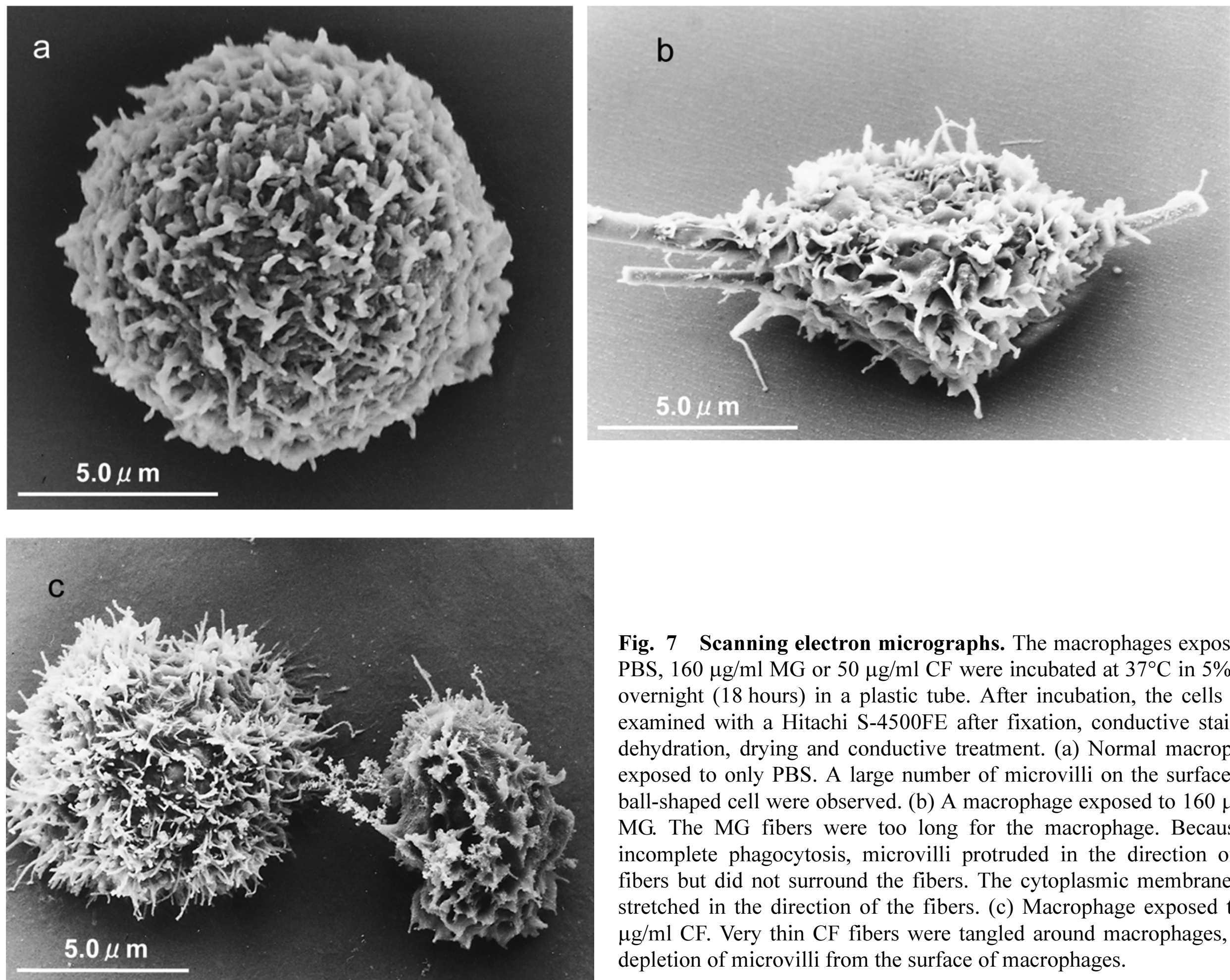

Fig. 7 Scanning electron micrographs. The macrophages exposed to PBS, 160 μg/ml MG or 50 μg/ml CF were incubated at 37°C in 5% CO_2 overnight (18 hours) in a plastic tube. After incubation, the cells were examined with a Hitachi S-4500FE after fixation, conductive staining, dehydration, drying and conductive treatment. (a) Normal macrophage exposed to only PBS. A large number of microvilli on the surface of a ball-shaped cell were observed. (b) A macrophage exposed to 160 μg/ml MG. The MG fibers were too long for the macrophage. Because of incomplete phagocytosis, microvilli protruded in the direction of the fibers but did not surround the fibers. The cytoplasmic membrane was stretched in the direction of the fibers. (c) Macrophage exposed to 50 μg/ml CF. Very thin CF fibers were tangled around macrophages, with depletion of microvilli from the surface of macrophages.

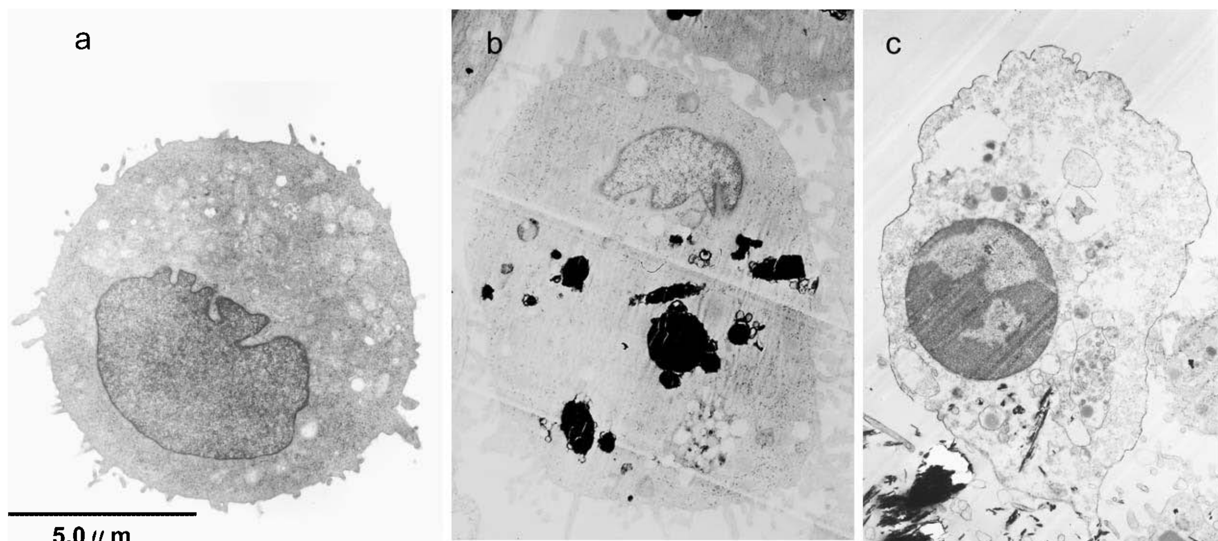

Fig. 8 Transmission electron micrographs. The macrophages exposed to PBS, 160 μg/ml MG or 50 μg/ml CF were incubated at 37°C in 5% CO_2 overnight (18 hours) in a plastic tube. After incubation, the cells were examined with a Hitachi H-600 after dehydration, resin embedding, ultrathin sectioning with an ultramicrotome and electron staining with uranyl acetate and lead citrate. (a) Normal macrophage exposed to only PBS. (b) A macrophage exposed to 160 μg/ml MG. Macrophage phagocytized MG fibers were observed in the cytoplasm, with vacuoles around them. (c) Macrophage exposed to 50 μg/ml CF. Vacuoles in the cytoplasm and nuclear condensation were observed in macrophage phagocytized CF fibers.

is suggested that the exposure to MG caused physical and/or chemical cytoskeleton damage, and the random rotation of phagosomes in the cell was inhibited. Morphological changes by TEM observed here were similar to cytoplasmic changes in macrophages which showed a decrease in the B_{20} (%) in previous studies (17, 18).

The enzymatic measurement of LDH, which is released from alveolar macrophages, has been used conventionally as an indicator of cellular membrane damage (11). A significant increase in LDH release from alveolar macrophages exposed to MG was observed in a dose-dependent manner in this study. In the study of Castranova (28), LDH were released extracellularly from rat alveolar macrophages in a dose-dependent manner due to the addition of short glass fibers, in agreement with our results. The exposure to MG also caused physical cellular membrane damage.

Physical properties are suggested to be involved in the cytotoxicity of fibrous materials (6). Martina et al. (29) suggested that the length of fibers that can be completely phagocytized is limited by the macrophage cell size. When fibers are too long to be completely engulfed by macrophages, oxidants and enzymes leak from the alveolar macrophages and cause cell damage. These cellular responses are defined as "frustrated phagocytosis". Frustrated phagocytosis contributes to the toxicity of long fibers such as MG. In the study of Castranova (28), the increased LDH released from the cells due to frustrated phagocytosis resulted in the leakage of oxidant and lysosomes from macrophages and they damaged the cells themselves. Ye et al. (30) examined cytotoxicity using two types of glass fibers of different lengths, and reported that short fibers could be phagocytized completely but long fibers could not be phagocytized. The consequent frustrated phagocytosis, caused by long fibers, induces the generation of active oxygen and the production of inflammatory and fibrogenic cytokines such as TNF-α, resulting in cytotoxicity. In morphological observation by SEM and immunofluorescent staining, some of the MG fibers were too long to be phagocytized completely by macrophages.

It is also suggested that the chemical composition of fibers contributes to their toxicity as much as the physical properties (31). Moyer et al. (32) suggested that the iron included in asbestos generates active oxygen through the Harber-Weiss reaction, which causes DNA injury and carcinogenesis. Although active oxygen species may also be generated in other minerals, the lesser iron content generates lesser amounts of active oxygen species, resulting in lower toxicity. The iron contents are lower in MG (0.41%) than in CF (5.0%). In this experiment, the cytotoxicity of MG was lower than that of CF. The cytotoxicity differences between MG and CF could be explained partly by the iron composition.

In the experiment of the effects of MG on macrophages by cell magnetometry, a significant decrease in the rate of relaxation was observed in a dose-dependent manner. A significant increase in LDH release was also observed in a dose-dependent manner. From the morphological observations using SEM and immunofluorescent staining, some macrophages engulf fibers incompletely (frustrated phagocytosis), and changes in the cytoskeletons were observed. Although its toxicity was not strong compared that of CF at the same concentration, it is important to reduce exposure to MG in the workplace. In the future, the toxicity of MG in the lungs should be examined *in vivo*.

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