Tohoku J. Exp. Med., 1998, **186**, 87-98

Magnetometric Evaluation for the Effect of Chrysotile on Alveolar Macrophages

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Department of Preventive Medicine and Public Health, Kitasato University School of Medicine, [1]Department of Public Health, Kitasato University School of Allied Health Sciences, [2]Department of Pathology, Kitasato University School of Medicine, Sagamihara 228-8555, and [3]Department of Electronics, Faculty of Engineering, Tokyo Denki University, Tokyo 101-0054

Keira, T., Okada, M., Katagiri, H., Aizawa, Y., Okayasu, I. and Kotani, M. *Magnetometric Evaluation for the Effect of Chrysotile on Alveolar Macrophages*. Tohoku J. Exp. Med., 1998, **186** (2), 87-98 —— Alveolar macrophages are thought to play an important role in fibrogenesis due to asbestos exposure. In this experiment, we evaluated the effect mainly by unique magnetometry and also by conventional methods such as lactate dehydrogenase (LDH) activity measurement and morphological observations. Alveolar macrophages obtained from Syrian golden hamsters by bronchoalveolar lavages were exposed 18 hours in vitro to Fe_3O_4 as an indicator for magnetometry and chrysotile for experiments. A rapid decrease of the remanent magnetic field, so called "relaxation", was observed after the cessation of an external magnetic field in macrophages phagocytizing Fe_3O_4 alone, while relaxation was delayed in those concurrently exposed to chrysotile. Since relaxation is thought due to the cytoskeleton-driven random rotation of phagosomes containing iron oxide particles, chrysotile is considered to interfere with the cytoskeletal function of macrophages. Release of LDH from chrysotile-exposed macrophages into the medium was recognized, but it was not significantly higher than the controls. Apoptosis was negligible in macrophages exposed to chrysotile by the DNA ladder detection, the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling method and morphological observations. Electron microscopical examinations revealed early necrotic changes in macrophages exposed to chrysotile. These findings indicate that cell magnetometry detects impaired cytoskeletal function due to in vitro exposure to chrysotile. —— magnetometry; chrysotile; alveolar macrophage; cytoskeleton; apoptosis

Received August 27, 1998; revision accepted for publication October 19, 1998.

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Because of their important role in fibrogenesis due to asbestos fibers, macrophages have been intensively investigated in cell cultures (IPCS 1986). There have been many reports on in vitro toxicity of asbestos (Koshi and Sakabe 1972; Gormley et al. 1980, 1983; Wright et al. 1986). Therefore, a sensitive method of evaluating toxicity of chrysotile and various man-made-mineral fibers will contribute to the assessment of safety and the application of these fibers.

Magnetometric evaluation of the effects of chemical substances to humans and animals were reported (Aizawa 1994; Moeller et al. 1996). However, cell magnetometry for evaluating the effects of chemicals to phagocytes has been only reported by the authors (Keira et al. 1996). This is the first report evaluating the effect of chrysotile on the alveolar macrophages by cell magnetometry.

In magnetometry, we evaluate the effect of chrysotile by relaxation, a rapid decrease of remanent magnetic field strength (RMF) radiated from phagocytized iron oxide particles in macrophages following the cessation of external magnetization (Cohen 1973). Relaxation is considered indicating the cytoskeletal function, because relaxation is thought to occur due to the random rotation of phagosomes driven by cytoskeleton (Valberg and Brain 1988; Nemoto et al. 1989).

Materiarls and Methods

Reagents

Fe_3O_4 particles with the mean geometric diameter of 0.26 μm (Toda Kogyo Company, Hiroshima) were used for an index of magnetometry. The standard sample B of International Union Against Cancer (UICC) was provided by Japan Fibrous Materials Research Association and used for the experiments of chrysotile exposure. The average short and long geometric diameters of the chrysotile fibers are 0.15 and 2.6 μm, respectively (Kohyama et al. 1996). These samples were suspended in phosphate-buffered saline (PBS) pH 7.4, then stirred by an ultrasonic generator. The fibers were washed twice by centrifugations and stirred just before use.

Bronchoalveolar lavage

A male golden hamster weighing about 100 g was anesthetized by an intraperitoneal administration of 100 mg/kg of pentobarbital sodium. The hamster was bled to death by an 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, then followed by gentle aspiration. This was repeated additional nine times.

Fluid from all ten lavages was pooled and centrifuged at 1800 rpm for 10 minutes. The cell pellet was suspended in Eagle's Minimum Essential Medium (MEM, Nissui Pharmaceutical Company, Tokyo) containing 10% fetal bovine serum (FBS). A part of the precipitate was stained by trypan blue and the viability of cells was always over 90%.

Cell culture

The cell suspension was adjusted to a concentration of 10^6/ml and 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 Fe_3O_4 particles suspended in PBS were added to all wells. For experiments, 20, 40 or 60 mg/ml of chrysotile fibers suspended in PBS were added to each of the experimental groups, while PBS was added for the controls. The groups were cultured at 37°C in 5% CO_2 for 18 hours. After incubation, a macrophage-adhered disk was removed from a well to a glass tube containing 1 ml of MEM with 10% FBS.

Magnetometry

The sample was magnetized by a magnetizer at 70 mT for 10 mseconds. Immediately after stopping magnetization, the remanent magnetic field was measured for 20 minutes by a fluxgate magnetometer (Institut Dr. Förster, Germany). As shown in Fig. 1, a sample tube was set to the stage rotating over the probe once every 6 seconds. In order to keep the temperature of air inside the magnetic shield at 37°C, the heater with a thermostat was used.

Time after magnetization was counted from zero when the magnetization was stopped, because iron oxide particles aligned completely in response to the exter-

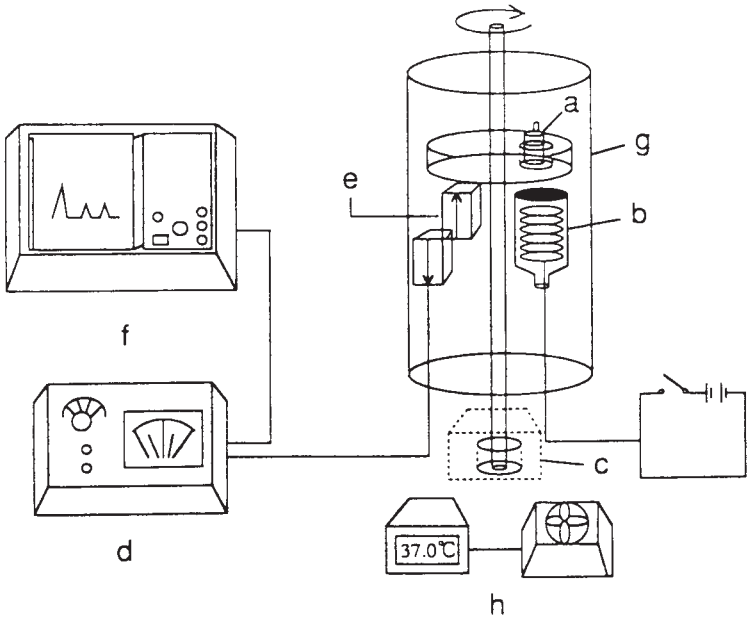

Fig. 1. Magnetometric apparatus. Macrophages adhering to the disk at the bottom of the glass tube (a) are magnetized at 70 mT for 10 mseconds by the magnetizer (b). After the cessation of external magnetization, the stage rotates by the motor (c). The RMF is measured every 6 seconds by the fluxgate magnetometer (d) with the probe (e) and recorded by the pen-recorder (f). The temperature inside the shield (g) is kept at 37°C by the heater (h).

nal magnetic field and started to misalign as soon as it was removed. A two-minute period of relaxation was fitted 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 minutes (Brain et al. 1988). Linear regression of natural logarithms of the 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

Alveolar macrophages exposed to 20, 40 or 60 μg/ml of chrysotile were incubated at 37°C in 5% CO_2 for 18 hours. The cell suspension was centrifuged at 1400 rpm for 10 minutes after incubation. The LDH activity in the cell-free supernatant was measured by Wroblewski-LaDue's method (Wroblewski and LaDue 1955) using the LDH-UV Test Wako (Wako Pure Chemical Industries, Osaka). In order to measure the 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; ([LDH from chrysotile-exposed cells]-[LDH from non-exposed cells]) × 100/(total LDH-[LDH from non-exposed cells]).

DNA ladder detecting method

Cultured macrophages exposed to 60 μg/ml of chrysotile or PBS for 18 hours were washed with PBS. For DNA extraction, 10 μl of each of proteinase K (Wako Pure Chemical Industries), RNase A (Wako Pure Chemical Industries) and 20 μl of 10% SDS (Wako Pure Chemical Industries) were added to the samples (Hagimoto et al. 1997). 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 freeze-dried until use.

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

DNA nick end-labeling

Cultured macrophages exposed to 20, 40 or 60 μg/ml of chrysotile or PBS for 18 hours were subjected to the terminal deoxynucleotidyltransferase (TdT) mediated dUTP-biotin nick end labeling (TUNEL) by using Apop Tag Plus In Situ Apoptosis Detection Kit (Oncor Co., Gaithersburg, MD, USA) with some modification. In order to inhibit endogenous peroxidase, hydrogen peroxidase and anti-digoxygenin-peroxidase were substituted for proteinase K and anti-digoxlgenin-alkaline phosphatase, respectively. For staining the samples, 5-bromo-4-chloro-3-indoxyl-phosphate and nitro blue tetrazolium chloride were used instead of diaminobenzidene substrate.

Four-micrometer-thick histologic sections of 10% buffered formalin-fixed and paraffin wax-embedded human tonsils were used for the positive controls.

Electron microscopy

Macrophages adhered on 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 the transmission electron microscopic (TEM) observation, the cells were examined with H-600 (HITACHI, Tokyo) after dehydration, resin embedding, ultra-thin sectioning and electron staining. For the observation under a scanning electron microscope (SEM), the cells were examined with S-4500FE (HITACHI) after ion spatter coating.

Statistical Analysis

Results are expressed as means±S.E. from six animals in each group. Statistical differences among group means were determined using one way or two-way analysis of variance and Scheffe's method.

Results

Magnetometry

The means of RMF obtained from macrophages exposed to chrysotile and PBS are plotted in Fig. 2. Relaxation was delayed in the groups exposed to chrysotile fibers compared with controls. Statistically significant differences were

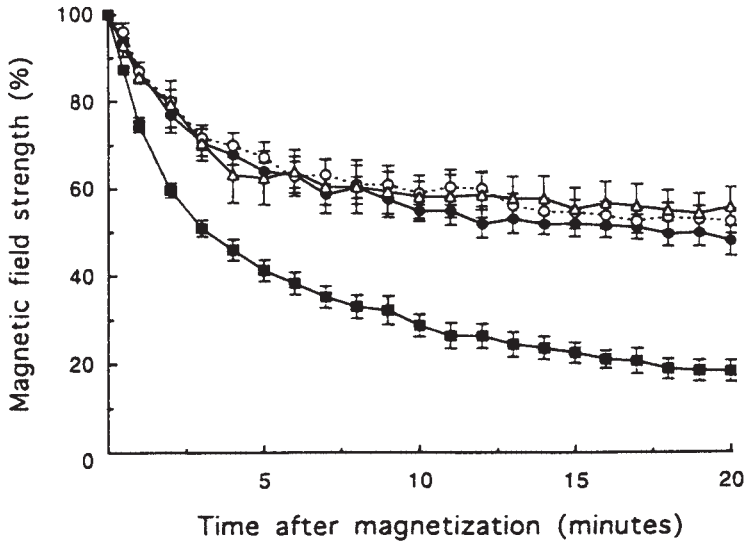

Fig. 2. Relaxation curves in the alveolar macrophages exposed in vitro to various doses of chrysotile fibers and PBS as controls. The normalized percent means of RMF with S.E. from 6 hamsters are plotted as the initial RMF as 100%. Significantly delayed relaxation curves were demonstrated in the groups exposed to 20 (●—●), 40 (○···○) and 60 μg/ml (△—△) compared with that of PBS-treated group as controls (■—■).

TABLE 1. *Decay constants, LDL release rates and proportion of apoptotic cells in alveolar macrophages exposed in vitro to chrysotile*

	Controls	Chrysotile (μg/ml)		
		20	40	60
Decay constant ($\times 10^{-3} \cdot sec^{-1}$)	4.49±0.32	2.34±0.35**	1.97±0.34**	2.22±0.34**
LDH release rate (%)	0.09±0.09	4.45±2.51	5.89±2.94	5.74±2.55
Apoptosis (%)	6.52±2.43	8.72±2.12	4.02±0.83	4.43±0.98

Values are means±S.E. from 6 hamsters in each experiment. **$p<0.01$ compared with the PBS-added control cells. No significant difference was found in LDH release rates nor in percentage of apotosis among groups.

found among the controls, the groups exposed to 20, 40 and 60 μg/ml of chrysotile by a two-way analysis of variance ($p<0.01$). As a result of a multiple comparison by Scheffe's method, significant differences at the 1% level were recognized between the controls and the chrysotile-exposed groups (Fig. 2).

The decay constants of the chrysotile-exposed groups showed a significantly lower value than that of the control group by a multiple comparison conducted under 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 macrophages exposed to various concentrations of chrysotile were slightly higher than that of controls as shown in Table 1, but the difference was not significant by an analysis of variance ($p=0.28$).

Apoptosis

No DNA fragmentation was found in the samples of chrysotile-exposed macrophages by the DNA ladder detection method (Fig. 3).

In the TUNEL method, cells with positive signals were scarcely observed under a light microscope (Fig. 4). Among 100 cells observed, the proportions of positive cells in chrysotile-exposed macrophages were not significantly different from the controls by the analysis of variance ($p=0.24$, Table 1).

Electron microscopy

Macrophages treated with PBS or Fe_3O_4 appeared intact (Figs. 5a and b), while those exposed to chrysotile were penetrated by fibers (Fig. 5c) in the SEM images.

Few cells demonstrated the disappearance of microvilli over the cell surface (Fig. 5c). The obvious destruction of cell membranes was not recognized.

In the TEM images, Fe_3O_4 particles and intact structure were observed in the

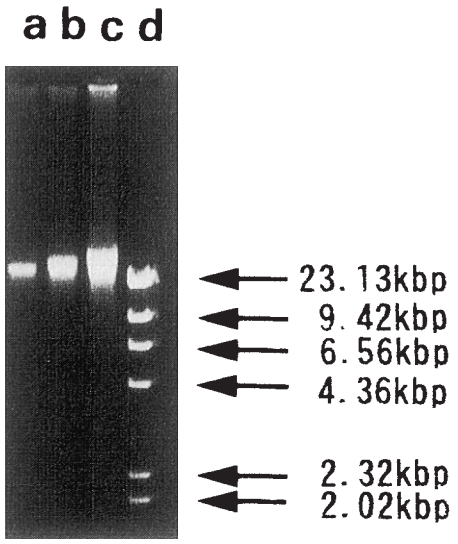

Fig. 3. Electrophoretic analysis of DNA exracted from alveolar macrophages. Macrophages exposed to 60 μg/ml of chrysotile for 18 hours (lane, a), PBS-added controls (lane, b), nothing-added controls (lane, c) did not demonstrate DNA fragmentation. Indicators with various killo-base pairs (kbp) were shown in lane d.

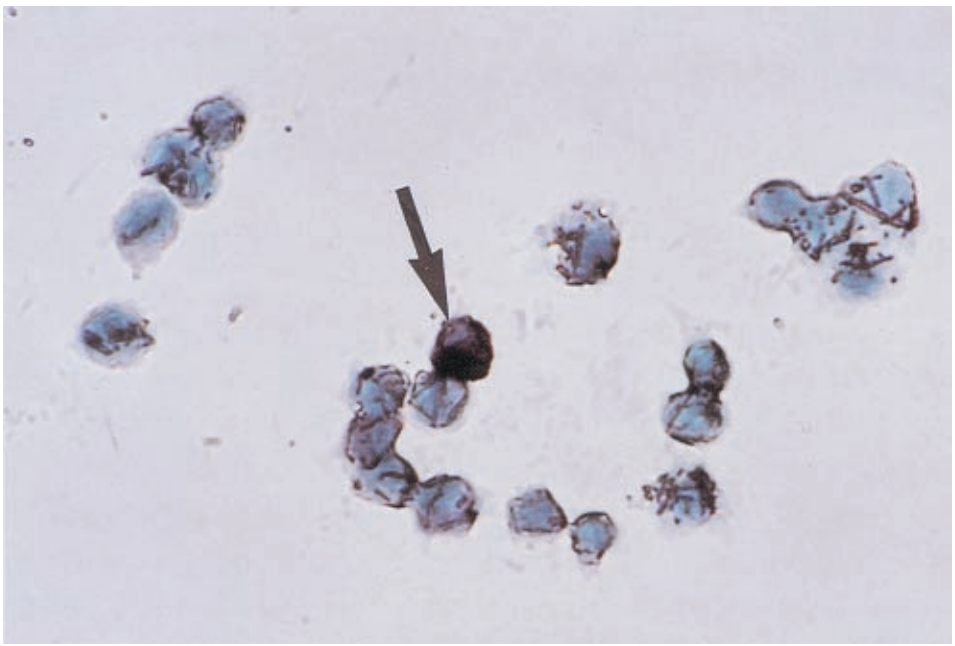

Fig. 4. Apoptosis in chrysotile-exposed alveolar macrophages detected by the TUNEL method. Macrophages exposed in vitro to 40 μg/ml of chrysotile for 18 hours were scarcely stained brown or black in nuclei. A cell pointed by an arrow indicates apoptosis. (Original magnification: $\times$200)

exposed macrophage (Fig. 5e) in comparison with the control (Fig. 5d). A plenty of chrysotile fibers, a number of vacuoles and blunt margins of mitochondria and rough endoplasmic reticulum were observed (Fig. 5f). No multiple nuclei with margination and condensation of the chromatin were observed, nor were shrinkage of cells or apoptotic bodies.

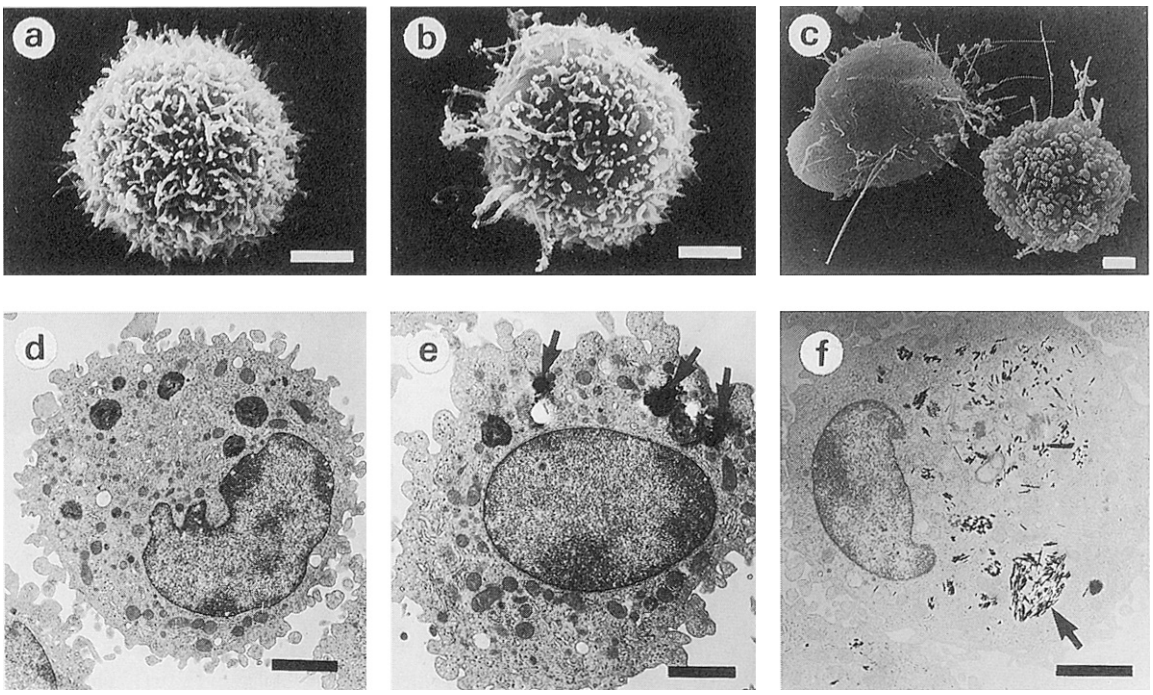

Fig. 5. The representative electron microscopic images. (a) A control macrophage by SEM. (b) A macrophage exposed to iron oxide particles by SEM. (c) Two macrophages phagocytizing chrysotile by SEM. One cell demonstrates the absence of microvilli. (d) A control macrophage by TEM. (e) TEM image of a macrophage phagocytizing iron oxide particles as arrows indicate. (f) TEM image of a macrophage phagocytizing a plenty of chrysotile fibers indicated by an arrow. Vacuoles and unclear margins of mitochondria and other organelles are observed. Each bar represents 2 μm.

Discussion

It has been reported that asbestos causes respiratory disorders such as asbestosis, pleural changes, lung cancer and mesothelioma in humans (Selikoff et al. 1980; Berry 1981; Finkelstein et al. 1981; Hillerdal 1981; Cookson et al. 1985). Chrysotile is known to be cytotoxic to cultured cells (Koshi and Sakabe 1972; Allison 1973; Gormley et al. 1980, 1983; Koshi et al. 1991). Alveolar macrophages are thought to initiate fibrotic processes in the development of pneumoconiosis when exposed to asbestos (Balmes and Scannell 1997).

Cell magnetometry is a modification of pneumomagnetometry conducted first by Cohen (1973; Cohen et al. 1979). The principles of these methods are to administer iron oxide particles in the lungs or cultured phagocytes and let alveolar phagocytes engulf them. External field magnetizes iron oxide particles located in phagosomes of macrophages (Brain et al. 1988; Valberg and Brain 1988). The RMF decreases rapidly after stopping external magnetization and this phenomenon is called relaxation. Relaxation is thought to occur due to the random rotation of phagosomes containing magnetized iron oxide particles (Cohen et al. 1979; Nemoto et al. 1989). Cytoskeleton is thought to contribute to the random rotation of phagosomes (Valberg and Brain 1988). Therefore, delayed relaxation

is considered to indicate the dysfunction of cytoskeleton due to exposed chemicals, though detailed mechanism is still to be elucidated.

In in vivo experiments, intratracheally instilled gallium arsenide in rabbits caused a delay in relaxation and it also caused diffuse chemical pneumonitis (Aizawa et al. 1994). Delayed relaxation was also induced by cigarette smoking (Moeller et al. 1996). On the other hand, relaxation was accelerated when rats were infected with the influenza virus (Fukushima et al. 1995) or their alveolar macrophages were exposed to interferon-r (Fukushima et al. 1994).

In in vitro experiment, there is only one previous report observing no effect of limestone to relaxation in alveolar macrophages obtained by BAL of hamsters (Keira et al. 1996). In the present paper, in vitro exposure of 20 μg/ml or higher doses of chrysotile 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.

LDH in the extracellular fluid indicates sublethal cytotoxicity. In the present study, LDH release rate was not as high as those reported earlier (Koshi and Sakabe 1972; Kaw et al. 1982). There are three reasons for this.

First, the exposure time of chrysotile was 18 hours in this study instead of 24 or 48 hours in other studies (Koshi and Sakabe 1972). When cells were exposed to chrysotile in the culture medium longer than 24 hours, the LDH release increased (Allison 1973).

Second, FBS was present in the culture medium used for chrysotile exposure. The surface of the fibers was coated by the serum, consequently toxicity of chrysotile to cell membranes was diminished (Allison 1973).

Last, the length of chrysotile fibers used for exposure to macrophages were considered shorter than original ones, because the samples were repeatedly stirred well in an ultrasonic generator. When the length of fibers gets shorter, the number of fibers completely penetrating cell membranes decreases. Short fibers were reported to produce a milder LDH release than longer fibers (Kaw et al. 1982).

As for apoptosis, no difference was observed between the controls and the chrysotile-exposed macrophages by the DNA ladder detection method and the TUNEL method. Short exposure time, presence of FBS and shortening of the length of fibers are thought contributing to negative apoptotic changes in this experiment, because a previous study observed an occurrence of apoptosis due to chrysotile exposure (Hamilton et al. 1996). The electron microscopical observation also indicated negligible apoptosis of macrophages exposed to chrysotile.

It has been known that cytoskeleton mainly consists of microtubules, microfilaments, and intermediate-diameter filaments. They play important roles in integrity of cytoplasm, transport of organelles, cell polarity and mobility of cells (Malorni et al. 1990; Cassimeris 1993; Maccioni and Cambiazo 1995; Brinkley 1997). 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. The rotation of phagosomes assessed by relaxation may indicate the cytoskeletal function. Therefore, relaxation could be a sensitive indicator of the effect of chemicals to cytoskeleton.

Light and Wei (1977) ascribed the hemolytic activity of chrysotile fibers to surface properties as manifested by the Zeta potential. Miller and Harington (1972) suggested that the cytotoxic activity of asbestos was due to the interaction of magnesium ions, situated on the surface of the fibers, with cell membrane glycolipids. However, the mechanism of impaired cytoskeletal function due to chrysotile has not been elucidated. The mechanical interaction of chrysotile with phagosomes and cytoskeletal system is possible hypothesis explaning the delayed relaxation.

In this study, during 18 hour exposure to chrysotile, a conspicuous change in alveolar macrophages developed in the cytoskeletal function, while changes of cell membranes indicated by LDH release as well as of nuclei shown by apoptosis tests were negligible. Therefore, cell magnetometry is thought to be a sensitive method to detect the effect of chrysotile on the alveolar macrophages.

Acknowledgment

The authors are grateful to Ms. K. Mashimo, Ms. Y. Sugiura, Ms. Y. Inoue, Mr. S. Miyazawa and Ms. N. Nemoto for their technical assistance. This study was supported by the Academic Frontier Project of the Ministry of Education, Science, Sports and Culture.

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