

Review Article

In Vitro Studies on Biological Effects of Fibrous Minerals

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Abstract: Asbestos substitutes have been used recently in industrial various applications. Since certain asbestos substitutes have similar characteristics of asbestos, they require urgent *in vitro* and *in vivo* evaluation of these asbestos substitutes prior to occupational applications. Though *in vitro* studies do not offer precise assessment of toxicity of the fibers, it is possible to provide useful information as to the biological effects of asbestos and its substitutes. This review articles described the findings of *in vitro* experiments in investigation of biological effects of asbestos and man made mineral fibers (MMMF) and their correlation with *in vivo* assays; 1. Cytotoxicity, geometry and dimension of fibers. 2. *In vitro* biological effects of fibers on a mass basis and a numerical basis. 3. Mechanism of cytotoxicity, carcinogenicity and cell proliferation including *in vitro* cytokines production. The relationships between the *in vitro* and the *in vivo* biological effects of fibers do not always coincide. Therefore, safety of the fibers must be assessed in both *in vivo* and *in vitro* using an inert fiber as negative control. Additionally, evaluation of safety of these fibers *in vitro* must be conducted in comparable concentrations, sizes and numbers of fibers for used in *in vivo* experiments.

Key words: Mineral fibers, Man made mineral fibers, Asbestos, *In vitro*, Biological effects

Abbreviation

TO:	titanium oxide whisker
IL:	Interleukin
TNF:	tumor necrosis factor
NK cell:	natural killer cell
LAK cell:	lymphokine-activated killer cell
LDH:	lactic dehydrogenase
GRO- α :	growth-regulated peptide-alpha
EGF:	epidermal growth factor
PDGF:	platelet-derived growth factor

Introduction

Recently, various asbestos substitutes have been used over a wide range of industrial applications. Since certain asbestos substitutes have similar characteristics to some form of asbestos, they raise concern for potential health effects for

workers exposed these fibers in occupational environments. The safety of asbestos substitutes generally called "man-made mineral fibers (MMMF)" has not been completely validated. It indicates that only a short term acute *in vitro* and *in vivo* effects of MMMF have been studied. While effects of asbestos on induction of mesothelioma, bronchogenic carcinoma and pulmonary fibrogenic effects have been confirmed in human, there is a time lag during 20–30 years before emergence of mesothelioma or lung carcinoma after the inhalation of asbestos. Therefore, this evidence strongly suggests that their safety is necessary to be evaluated immediately before occupational application of MMMF *in vitro* and *in vivo* experiments.

Large quantities of well characterized chemical components of defined asbestos substitutes are commonly used in test inhalation models in animals for risk assessment and/or investigation of the effects of asbestos. Additionally, asbestos-induced carcinogenic effects have been observed

during 2–3 year experimental period using a large number of animals, animals care facilities, manpower and a high costs for prolonged animal care for *in vivo* experiments.

On the other hand, *in vitro* studies allow us to evaluate cytotoxicity, mutagenicity and carcinogenic potential of asbestos and MMMF in relatively short time. At first, the asbestos-induced cell damage was investigated using red blood cells and later with an established cell line in cell cultures and cells obtained directly from the respiratory tract. More recently, organ cell culture, primary cell culture, various cell line and bacterial system were added in the *in vitro* methods for use with various recent molecular biological techniques. These *in vitro* techniques are especially useful for studying the mechanism of cytotoxicity, cell proliferation, morphologic transformation induced by fibers. These pathological changes are attributed to various fiber-associated lung diseases, such as fiber-induced inflammation, pulmonary fibrosis, mesothelioma and lung carcinogenesis. However, one must recognize limitations of *in vitro* studies as they fail to elucidate certain important factors determining respirability, deposition, retention, and clearance of fibers in the respiratory tract. The information derived from *in vitro* experiments is also an important to understand the physical effects of fibers, including their length, diameter, density, and surface characteristics and chemical structure. Though *in vitro* studies can not accurately assess toxicity of the fibers, it is possible to provide useful information as to the biological effects of asbestos and its substitutes.

This review describes the findings of *in vitro* experiments in investigation of biological effects of asbestos and MMMF and their correlation with *in vivo* assays.

The Relationship among Cytotoxicity, Geometry and Dimension of Fibers

A few experiments reported that longer, thinner fibers (i.e., those with lengths >8–10 μm and diameters <0.25 μm) are more efficient in exerting biological effects as in *in vitro* such as induction of ornithine decarboxylase than the short fibers (lengths <0.25 μm) or nonfibrous particles¹⁻³. When

natural fibers were with synthetic mineral fibers, Chamberlain *et al.*^{4,5} reported that cytotoxicity of both attapulgite and sepiolite fibers is directly related to the length fiber using the cultures of human lung carcinoma and Chinese hamster lung fibroblasts (V79-4). In response of pulmonary alveolar macrophages to grass fibers, Ultra-fine (length 5.0–19 μm 55%, and diameters <0.1 μm 63.7%) fibers showed more cytotoxic activity in lactic dehydrogenase (LDH) secretion⁶ from pulmonary alveolar macrophages in response to exposure to glass fibers. Inhibition of growth or release of cytoplasmic enzymes was not observed in macrophage-like cells⁷ and hepatocytes⁸ after incubation with short fibers shorter than 1.0 micrometer (both attapulgite and xonolite). Relating to the *in vitro* findings, Pott⁹ and Stanton *et al.*¹⁰⁻¹² have reported the correlation of fiber sizes and the induction of mesotheliomas and plural sarcomas in experimental animals. Both intraperitoneal and intrapleural injection of asbestos resulted that fibers longer than 8 micrometer with diameters of less than 0.25 micrometer show marked carcinogenic potential. This finding is supported by the report of Davis¹³ (1989) in inhalation studies using long and short (<5 μm length) preparations of chrysotile and amosite asbestos administered to rats. Davis¹³ appears to have demonstrated a better correlation between the dimension of fibers *in vitro* and the *in vivo* findings than other investigators.

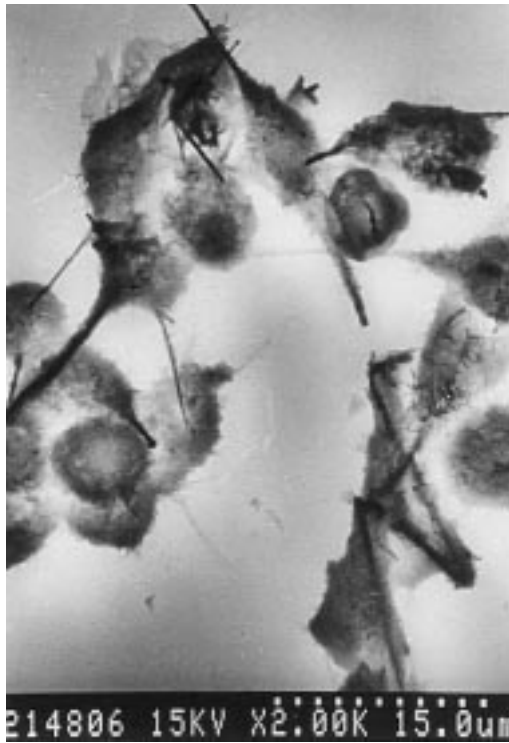
Comparison of *In Vitro* Biological Effects of Fibers on a Mass Basis and a Numerical Basis

A difficulty in the interpretation of *in vitro* studies is the introduction of fibers on based on mass (milligram of fibers per dish) rather than on the number (numbers of fibers per dish)¹⁴. Table 1 and Fig. 1 describe the geometric and physical dimensions of whisker test materials on murine macrophages cell line, RAW264.7 cells¹⁵. As results shown in Figs. 2 and 3, the three types of whiskers caused slight increase in lactic dehydrogenase (LDH) transudation and a decrease of cell viability in 24 h of incubation in a dose-dependent manner compared with the control level. There

Table 1. Geometric dimensions of the test materials

Sample	Mean length (μm)	Standard deviation	Mean width (μm)	Standard deviation	Range of length (μm)	Range of width (μm)
TiO ₂ whisker	2.10	2.00	0.14	1.53	0.5– 20	0.05–1.00
K ₂ Ti ₈ O ₁₇ whisker	6.00	2.04	0.35	1.51	0.5–100	0.05–1.00
SiC whisker	6.40	2.45	0.30	1.58	0.5– 40	0.05–1.00
UICC crocidolite	2.10	0.30	0.12	0.01	0.5– 80	0.05–1.00

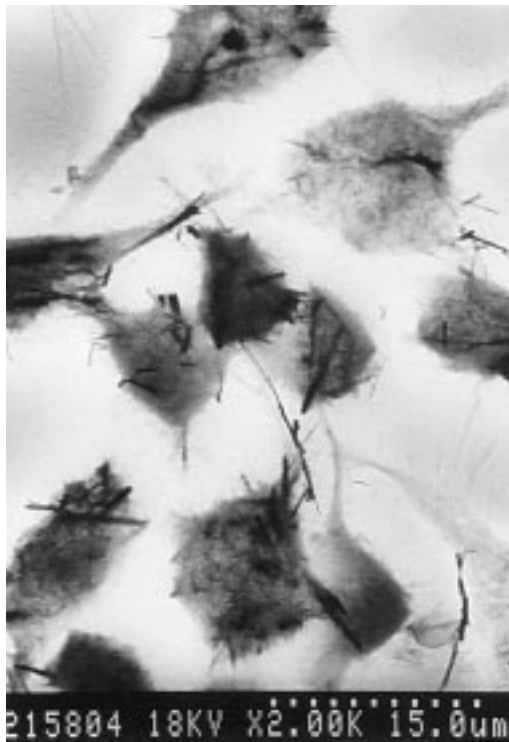
(a) SC1



(b) PT1



(c) TO1



(d) Crocidolite

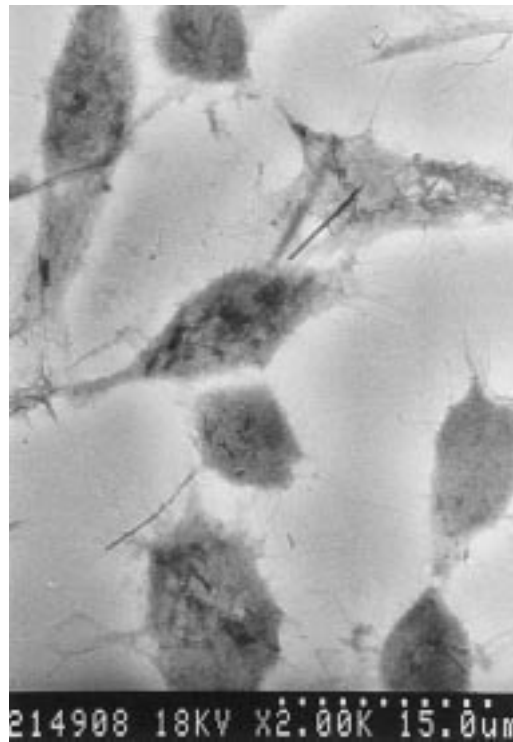


Fig. 1. Scanning electron micrographs of RAW 264.7 cells.

Murine macrophages ingested SC1, PT1, TO1 and crocidolite fibers (100 $\mu\text{g}/\text{ml}$). Cells were incubated with fibers for 24 h. Fiber-ingested cells are visualized by negative back-scattered electron imaging micrographs ($\times 2000$). PT1: $\text{K}_2\text{Ti}_8\text{O}_{17}$ whisker, SC1: SiC whisker, TO1: TiO_2 whisker.

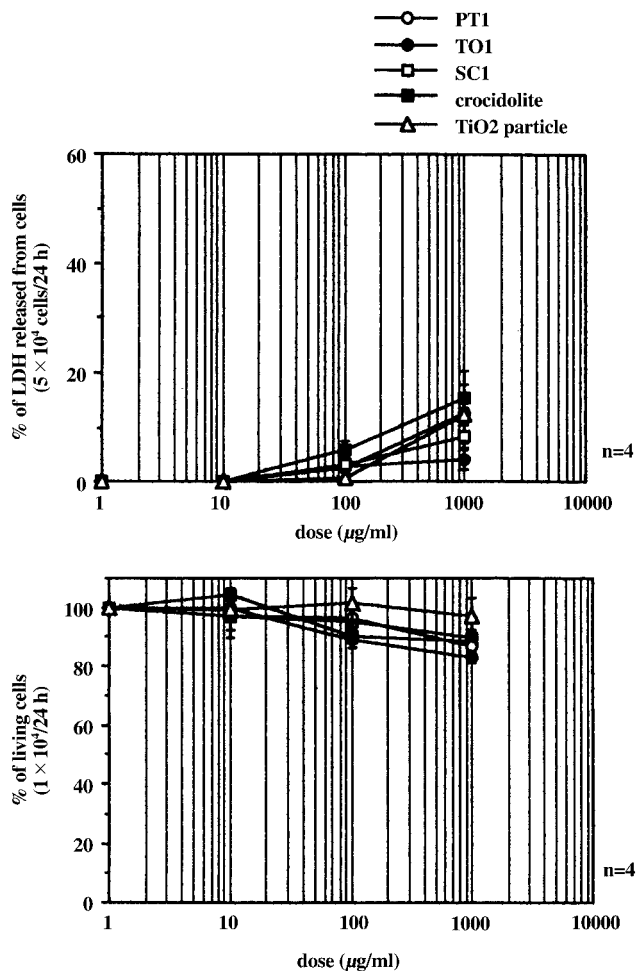


Fig. 2. Dose-response curves of LDH transudation from cells (upper graph) and cell viability (lower graph) after 24 h of incubation with test materials in RAW 246.7 cells.

PT1: $K_2Ti_8O_{17}$ whisker, SC1: SiC whisker, TO1: TiO_2 whisker.

were no significant differences among the three whisker materials. TNF- α levels in the culture medium attained a plateau at 12 hour in a dose-dependent manner up to 0.1 mg/ml concentration of whiskers, and titanium oxide whisker (TO1) induced about double the TNF- α of other whiskers. At 2 month after a single intratracheal injection at high dose into rats, PT1, TO1 and SC1 caused a substantial aggregation of inflammatory cells around the deposited fibers. This caused a very slight fibrous reactions regardless of the type of whiskers.

When biological effects of fibers are compared on the basis of number per unit mass, the three whiskers showed very similar concentration profiles with respect to TNF- α production (Table 2). From these results, we concluded that whisker-induced biological effects may be caused by the shape and size of the fibers and not attributable to their

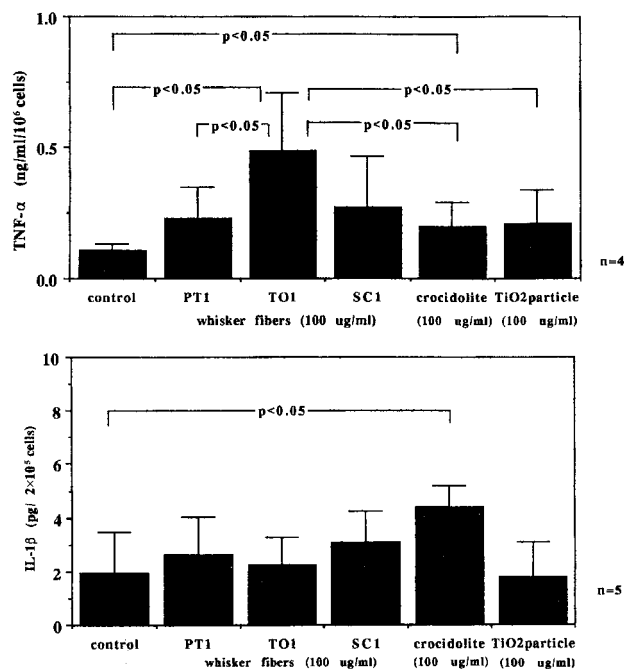


Fig. 3. Comparison of effects of three types of whiskers, crocidolite, and TiO_2 particles ($100 \mu g/ml$) on TNF- α concentration in culture medium (upper graph) and intracellular contents of IL- 1β (lower graph) at 12 h of incubation with materials, $37^\circ C$ in CO_2 incubator. Cells adhered in each well at a density of 2×10^5 cells/ $100 \mu l$ medium. PT1: $K_2Ti_8O_{17}$ whisker, SC1: SiC whisker, TO1: TiO_2 whisker.

chemical composition. TNF- α production could be associated with the number of the whiskers incorporated in macrophage cells. Additionally, the differences of the three whiskers in size and number of fibers per unit mass did not reflect the degree of inflammation and fibrosis in the lungs of the whisker-treated rats.

Palekar *et al.*¹⁶⁾ also evaluated cytotoxic effects of chrysotile, crocidolite, and two samples of erionite fibers, comparing with V79 fibroblasts using both mass basis and a numerical basis. No difference in cytotoxicity was observed between groups when fibers were evaluated on a mass basis, but both samples of erionite were several orders of magnitude more potent in causing cell death than crocidolite. A fiber of intermediate potency, i.e. chrysotile was the least toxic, when equal number of fibers was used in the evaluation. These results correlate with the higher tumorigenic potential of erionite in comparison with both types of asbestos in rodent inhalation experiments¹⁷⁾. These evidence suggest that it is important to evaluate biological effects of fibers on both mass and number basis. It is also necessary to confirm the *in vitro* findings with *in vivo* studies.

Table 2. Comparison of biological activities among the test materials in RAW 264.7 cells

Item	Total number ^a ($\times 10^6/\mu\text{g}$)	Biological activity of basis on number (1×10^6 fibers)			
		TNF- α (ng)	IL-1 β (pg)	LDH transudation (%)	Cell viability (%)
SC1	0.38	0.55 \pm 0.22	8.14 \pm 2.95	8.4 \pm 3.9	87.9 \pm 5.5
TO1	0.63	0.50 \pm 0.25	3.56 \pm 1.70 ^b	4.4 \pm 2.2	83.2 \pm 4.9
PT1	0.35	0.50 \pm 0.16	7.60 \pm 3.97	6.9 \pm 7.7	90.6 \pm 11.1
Crocidolite	0.90	0.23 \pm 0.17	4.85 \pm 0.89	6.3 \pm 1.9	88.9 \pm 1.9

^aScanning electron microscopy was used to analyze fibers, and data were provided by Dr. Yamato of University of Occupational and Environmental Health, Japan. ^bSignificant difference at $p < .05$ vs. SC1 and PT1.

Mechanisms of Cytotoxicity, Carcinogenicity and Cell Proliferation on *In Vitro* Studies

Generation and release of reactive oxygen species (ROS) from cells incubated with fibers

There are numerous publication in recent reporting that asbestos induce reactive oxygen species (ROS) and, its role in fiber-induced cell damage^{17, 18}. These reactive oxygen species appear to be second messengers in asbestos-elicited cell damage which involves increased membrane fluidity, lipid peroxidation^{19, 20} and breakage of DNA^{21–23}.

Hansen *et al.*²⁴ reported that fibrous dusts (defined as a greater than 3:1 of length: diameter ratio) caused a significant increase in both release of superoxide from rat macrophages and enhancement of zymosan-triggered superoxide production in hamster macrophages. Nonfibrous particles were less active than fibers at comparable concentrations. These results suggest that whereas short fibers and particles are incorporated into phagolysosomes, longer fibers are less likely to be phagocytosed by cells *in vitro*. Alveolar macrophage challenged with asbestos fibers produce a rapid release of superoxide anions²⁵, this indicate to result from protein kinase C activation by phospholipase C²⁰, or from the cross-linking of Fc receptors on the cell surface when challenged by IgG-opsinized fibers²⁶.

Hill *et al.*²⁷ reported that superoxide generation in isolated rat alveolar macrophages was enhanced by IgG coating of respirable fibers. Though contribution of ROS on cytotoxicity is still unclear, MMMF mediated release superoxide anion from macrophages appears to have less significant activity than that of asbestos-induced oxygen free radical. However, treatment with superoxide dismutase as a scavenger of superoxide anion fail to prevent cytotoxicity of glass fibers in tracheobronchial epithelial cells²⁸.

With respect to other asbestos-induced free radicals, Quinlan *et al.*²⁹ reported that asbestos induced induction of inducible nitric oxide synthase (iNOS) in alveolar

macrophages resulted elevation of nitric oxide production in both inhalation and *in vitro* models. Peroxynitrite formed by a reaction between nitric oxide and superoxide anion caused oxidant injury or after formation of hydroxyl-like radical³⁰. Such oxidant mediated cell injury may have been caused by a hydroxy-like radical. However, contradictory data suggested that nitric oxide is either protective or cytotoxic in acute and chronic phase of inflammatory response, modulation of cytotaxis, cell growth and differentiation²⁹.

Extracellular mechanism of ROS generation is also important with respect to various free radicals formed by interaction of asbestos fibers in cell-free solutions of H₂O₂ or physiological saline generate superoxide radicals by redox reactions on the fiber surface^{31–33}.

Carcinogenesis of asbestos and MMMF in in vitro studies

The carcinogenic potential of fibers is predicted by various *in vitro* tests such as chromosome mutagenicity, morphological and neoplastic transformation, gene mutation, etc. in the mammalian and bacteria cells *in vitro* studies. Sheby³⁴ reported that asbestos and conjugated estrogen were not mutagenic in both the Ames test using Salmonella and the rodent bone marrow bioassay which test chromosomal aberrations and micronucleated erythrocytes within 23 agents designated as IARC (International Agency for Research on Cancer) Group I human carcinogens.

On the other hand, in mutation tests using auxotrophic strains of Escherichia coli and Salmonella typhimurium Ames test, no mutagenic activity was found to associated with any of the asbestos and glass fibers tested over a wide range of concentrations³⁵. There was no mutagenic activity associated with three forms of asbestos in liver epithelial cells³⁶.

Kawauchi³⁷ reported that the correlation coefficient is 0.43 between the Ames test of mutation using Salmonella and concentration of 50%-induced carcinogenicity (IC50) in the 40 chemical mutants in experimental animals. There

is the 10^6 times difference between carcinogenic and mutation potentials, because carcinogenesis and tumor growth are not necessarily caused by mutation of DNA alone but also by losing regulation of cell cycles and intercellular communications. GJIC and carcinogenesis is more correlated than using Ames test³⁸).

Barrett's group^{39, 40} reported that transformation of mammalian cells in culture better correlated with fiber dimension. They demonstrated that the cytogenetic effects of asbestos and glass fibers on Syrian hamster embryo cells *in vitro* at doses which induced morphological and neoplastic transformation but which failed to induce measurable gene mutations in the cells at two genetic loci. Chrysotile asbestos treatment of the cells significantly induced chromosome changes in a dose-dependent manner, showing up to 50% of the cells had chromosome abnormalities in number or in structure, following treatment with $2.0 \mu\text{g}/\text{sq. cm}$ of asbestos for 48 hr. Treatment of the cells with thin glass fibers (Code 100) was also able to induce cell transformation and cytogenetic effects, but thick glass fibers (Code 110) were much less potent for both endpoints. However, there is difference between Barrett's group and other laboratories^{41, 42} in the frequency of transformation induced by asbestos and glass fibers in Syrian hamster embryo cells.

On the other hand, thirteen samples of natural fibers and five samples of MMMF were tested to determine their cytotoxicity and ability to produce chromosome missegregation in rat pleural mesothelial cells *in vitro*⁴³. Cytotoxicity appears to be dependent on both length and diameter of the fiber, where the longest or thickest fibers were the most toxic. The production of abnormal anaphases/telophases appears to depend on the presence of fibers of selected size, such as those previously defined by Stanton hypothesis. When compared with *in vivo* animal findings, there was no correlation between cytotoxicity and mesothelioma induction by the fibers, whereas a correlation was found between the ability of a sample to produce chromosome missegregation *in vitro* and mesothelioma *in vivo*. Recent *in vitro* studies demonstrated fiber induced carcinogenesis in human mesothelioma cell line and amniotic fluid cells by using molecular biologic techniques⁴⁴⁻⁴⁶. In rodent fibroblast cell lines, there is report that glass, erionite and potassium octatitanate fibers caused morphologic transformation⁴⁷. Ollikainen *et al.*⁴⁸ reported that asbestos fibers induced DNA single strand breaks in human mesothelial cells *in vitro*. Their results also suggested that antioxidant enzyme (SOD) did not have an affect on the DNA damage caused by the fibers, other mechanisms than free radicals seem to be involved in the induction of DNA

damage by mineral fibers.

As early as 1988, fiber-induced oxidative DNA damage were reported by assaying with 8-hydroxydeoxyguanosine (8-OH-dG) which causes mutations *in vitro* in mesothelioma cells and J774 cells⁴⁹⁻⁵¹. Investigators reported that this could be a better marker of oxidative DNA damage in describing potentially mutagenic lesions in DNA and/or apoptosis, despite compensatory increases in expression of an antioxidant enzyme. Some antioxidant enzyme and agents, i.e. SOD, catalase, mannitol or desferroxamine failed to inhibit action of ROS and iron ions as a source of Fenton reaction released from rat liver microsomes incubated with asbestos and NADPH⁵².

Studies of cell proliferation on asbestos- and MMMF-induced carcinoma and pulmonary fibrosis

Asbestos is a tumor promoter and a trigger of pulmonary fibrosis according with acceleration of cell proliferation as like epithelial and mesenchymal cell populations leading to the development of bronchogenic carcinoma, and asbestosis. Both evidences are confirmed *in vitro*⁵³ and *in vivo*⁵⁴ studies. These proliferative events can be measured by incorporation of tritiated thymidine⁵⁵, or bromodeoxyuridine⁵⁶ into DNA, or by immunohistochemical staining of proliferating cell nuclear antigen (PCNA)⁵⁷. All the techniques show the similar results, i.e. low background proliferative rates in the lungs ($\approx 1\%$), and 5- to 30-fold increases in percentages of dividing cells, depending upon the cell types, proximity to lung injury, and time after exposure.

The induction of cell proliferation by various types of asbestos and MMMF in cell line and organ cultures of rodent tracheobronchial epithelial cells. Woodworth *et al.*⁵⁸ found that induction of the squamous metaplasia in response to synthetic fibers in organ cultures of hamster trachea. When assay with biological concentrations on a mass basis, less $8 \mu\text{m}$ of length fibers in both asbestos and MMMF cause enhanced incorporation of tritiated thymidine, increased biothynthesis of polyamines and increased amounts of squamous metaplasia and keratinization in culture of trachea. Fiber of less $2 \mu\text{m}$ of length must be introduces at several-fold higher amounts than long fibers to achieve these effect, because nonfibrous particles do not cause any changes. Therefore, this evidence indicated that the bioassay have been useful in determining the characteristics of fibers important in causing altered proliferation of epithelial cells, the progenitors of bronchogenic carcinoma. Their results is better correlation compared with cytotoxicity and transformations assays.

However, mechanism of the process of malignant transformation of mesothelial cells *in vitro*, questions about the similarities and differences of mechanisms of spontaneous and asbestos-induced transformation remain unsolved⁵⁹. Because rat pleural mesothelial cells of early passages are different from the majority of epithelial and mesenchymal cells in their response to epidermal growth factor (EGF). However, EGF stimulates proliferation of rat mesothelial cells transformed *in vitro* by asbestos⁶⁰, and they were able to secrete EGF-like growth factors by themselves which are not synthesized by normal rat mesothelial cells⁶¹. From these evidences, we still need to explore the role of various growth factors, which are important for proliferation of cell lines, for mesothelial cells. Concerning the role of growth factors in the process of rat mesothelial neoplastic transformation, Kravchenko *et al.*⁵⁹ reported spontaneous and asbestos-induced transformation of mesothelial cells *in vitro* using rat pleural mesothelial cells. Although EGF began to activate mesothelial cell proliferation from the first stage of transformation, the addition of this growth factor did not facilitated these cells to grow in semisolid media either at the first or at the second stage. This indicates that mitogenic effect of EGF by itself was insufficient for transformation, and autocrine secretion of this growth factor by mesothelioma cells could not cause the appearance of some important properties of malignant cells. Their results proposed the necessity of a positive EGF response for mesothelial cell transformation and the similarity of mechanisms of spontaneous and asbestos-induced transformation.

With respect to exogenous fibers and proliferation of fibroblasts, Fisher *et al.*⁶² reported the relationship between doses ($0.16 \mu\text{g}/\text{cm}^2$ to $1.32 \mu\text{g}/\text{cm}^2$) of asbestos and glass fiber and growth and viability of Syrian hamster diploid embryonic fibroblasts and Chinese hamster peritoneal fibroblasts (B14F28). In both cell types tested, the toxic effects of asbestos and glass fibers at high doses increased in a concentration-dependent manner, i.e. colony numbers decreased. The stimulation of proliferation by low doses of particulate dusts, which could be demonstrated in B14F28 cells by the emergence of bigger colonies, corresponding to higher cell numbers per culture, and in the case of primary fibroblasts by increased colony counts. There was a threshold below which no activity could be detected. Since the supernatants of fiber-treated cells were able to enhance cell growth after removal of the dusts by filtration, they suggested that the activity was due to autostimulatory factors released by the cells.

Production of various cytokine on asbestos and MMMF stimulation in in vitro studies

Alveolar macrophages play a pivotal role in the host defense involving inflammatory response in the lung from inhaled asbestos fibres, and in the pathogenesis of asbestos-related disease, notably by producing cytokines and free radicals⁶³⁻⁶⁵. Cytokines involving chemokines and growth factors are deployed in both acute and chronic stage of chronic stage of inflammation, carcinogenesis and immune responses, etc. These cytokines have variety of biological effects, their roles are important in the process of diseases development. Asbestos is also capable of stimulating production and release of various cytokines from macrophages, fibroblasts and tumor cells. As our experimental results⁶⁶ (Table 3 and Table 4) indicate that there are changes in the levels of various cytokines in human alveolar macrophages treated with asbestos *in vitro*, and in macrophage-like cell line treated with MMMF *in vitro*. The results shown in these tables also describe cytokine production on a mass basis. Human alveolar macrophages release TNF- α , IL-1 β , IL-8, MIP-1 α , growth-regulated peptide (GRO- α) upon stimulation with asbestos and MMMF. In addition to these cytokines, asbestos also induce production and release of IL-6 in both human and rat macrophages⁶⁷⁻⁶⁹.

TNF plays a critical role in normal host resistance to infections and to malignant tumor growth, serving as an immunostimulant and as a mediator of inflammatory response. TNF- α is produced by neutrophils, activated T and B lymphocytes, NK cells, LAK cells, endothelial cells, smooth muscle cells, and some transformed cells⁷⁰. Many of biological effects of TNF are functionally similar to those of IL-1. Inhibition of TNF prevents the development of pulmonary fibrosis *in vivo* in animals investigated for asbestos- and silica-induced pulmonary fibrosis^{71,72}. These evidences indicate that macrophage-derived TNF- α is involved with asbestos- and MMMF-induced pulmonary fibrosis. However, there is inconsistency between the amount of TNF production in alveolar macrophages when stimulated with asbestos. Perkins *et al.*⁷³ showed that normal human alveolar macrophages release a very small amount of TNF, but they do not make significant amount of cytokines when the cells are exposed to asbestos *in vitro*, whereas Zhang *et al.*⁷⁴ reported that both human alveolar macrophages and monocytes released large amounts of TNF and IL-6 after *in vitro* exposure to asbestos. Reasons for the differences in these studies is not known. There is one possible explanation that collection of human alveolar macrophages by the bronchoscope may have influence the quality of the specimen.

Although IL-1 is not produced by the pulmonary cells of

Table 3. Comparison of biological effects of various cytokine production and release in human alveolar macrophages treated with asbestos

Cytokines		amosite	crocidolite	chrysotile
TNF- α	intracellular			±
	extracellular		±	±
IL-1 β	intracellular		±	±
	extracellular		±	
IL-8	intracellular			±
	extracellular	±	±	±
MIP-1 α	intracellular			±
	extracellular		±	±
GRO- α	intracellular			±
	extracellular			±
FGF	intracellular	±	±	±
	extracellular	±	±	±
TGF- β 1	intracellular	±	±	±
	extracellular	±	±	±
TGF- β 2	intracellular	±	±	±
	extracellular	±	±	±

healthy individuals, in response to stimuli such as those produced by inflammatory agents, infections microorganisms, or microbial endotoxins. IL-1 plays important roles in immune functions on macrophages/monocytes, and lymphocytes, and acts on macrophages/monocytes. IL-1 is synthesized by these cells along with TNF and IL-6.

Various chemokines also play an important role in chemotaxis and activation of leukocytes in inflammation. The term chemokine refers to a superfamily of a dozen or so of small (8–10 kDa), inducible, secreted, pro-inflammatory cytokines. Therefore, evidence for the release of IL-8, MIP-1 α and GRO- α from macrophages upon stimulation with fibers in vitro may reflect modulation of acute inflammatory response in the lung induced by inhaled of asbestos and MMMF *in vivo*.

On the other hand, cell growth factors including TNF- α , platelet-derived growth factor (PDGF), insulin-like platelet derived growth factor, insulin-like growth factor type 1, transforming growth factor β 2 (TGF- β 2) are listed as responsible makers for cell proliferation. TGF- β as anti-proliferative agent mediates the formation of extracellular matrix, generally with activities that stimulate formation, and inhibit degradation of extracellular matrix. This sounds contradictory to the word “anti-proliferative”. It also acts as a potent suppresser of the immune response by suppressing differentiation of lymphocytes and cytotoxic T cell, NK cell and LAK cell. PDGF was originally discovered in serum

Table 4. The effects of MMMFs on production on TNF- α and IL-1 β in RAW 264.7 cells

Test materials	TNF- α	IL-1 β
Ceramic Fiber-F		
Ceramic Fiber-FS		
Ceramic Fibre-M		
micro Glass Wool	±	±
K-Ti-O Whisker		±
TiO ₂ Whisker		±
SiC Whisker		±

Ceramic Fiber-F: Si-Al ceramic fiber, amorphous, crushed at 400 kg/cm² containing hott particles, (Fiber Frax). Ceramic Fiber-FS: Si-Al ceramic fiber, amorphous, crushed at 400 kg/cm², and shott particles separated by sedimentation method, (Fiber Frax). Ceramic Fiber-M: Si-Al ceramic fiber, crystalline, crushsd at 400 kg/cm², (Fiber Max). Micro Glass Wool: Glasswool with fine diameter (1–3 μ m), amorphous, crushed at 15 t/incn2, (Manvill #100). K-Ti-O Whisker: Potasium titanium oxide whisker, made by Otsuka Chemical Co. TiO₂ Whisker: Titanium oxide (rutile) whisker, made by Ishihara Sangyo Co., (FTL-300). SiC Whisker: Silicon carbide whisker, made by Tokai Carbon Co.

as the major mitogenic activity responsible for the growth of cultured mesenchymal cells. It is well known that PDGF is derived from platelet, PDGF-like factor derived from macrophages also act as a chemotactic and growth-stimulatory agent for smooth muscle cells⁷⁵. GRO- α was initially discovered as a product of a growth-regulating gene that is overexpressed constitutively in tumor cells. The same gene is transcribed in normal cells only during growth stimulation. GRO- α expression is inducible in vitro by serum or PDGF and/or by a variety of pro-inflammatory mediators, such as IL-1, TNF in monocytes, fibroblasts, mammalian epithelial cells. GRO- α is one of neutrophil-activating factors, can bind with high affinity to the type II IL-8 receptors⁷⁶.

In present our results, though we fail detection of cell growth factors without GRO- α in macrophages, Bonner *et al.*^{77,78} reported that asbestos stimulates the upregulation of PDGF- α in primary rat lung fibroblasts culture and that asbestos-stimulated macrophages to produced increased amounts of PDGF. Walker *et al.*⁷⁹ determined that the proliferation of asbestos-transformed mesothelial cells is at least partially regulated through a TGF- α -mediated autocrine feedback mechanism.

From molecular biology, cytokine expression in the cells is elicited through activation of NF- κ B in epithelial cells

and macrophages, and by activation of p38 kinase which has been shown to be important in the translation of cytokine message into protein⁸⁰⁻⁸²). The roles of various cytokines in asbestos- and MMMF-induced pulmonary disease is unclear yet. Additionally, various pulmonary cells products and releases various cytokines, they seems to play important roles in cell to cell interactions; i.e. macrophages, fibroblasts and epithelial cells during the progression of chronic diseases. Further experiments will be required to elucidate the mechanisms for cellular interaction of various pulmonary cells; which further influence expression of growth factors and cytokines *in vivo*.

Future Directions

The *in vitro* models have been valuable in defining mechanisms of interaction between fibers and monolayer cells. We also described that dimensions of fibers are important in eliciting these biologic responses. However, the crude dosimetry and poorly defined characteristics of fibers used in these studies did not allow precise comparison of individual fiber types over a wide range of concentration. Palker *et al.*¹⁶) suggest that fiber cytotoxicity compared on a number basis of fibers can be used to predict the pathogenicity of fibers. However, the relationships between the *in vitro* and the *in vivo* biological effects of the fibers do not always coincide. Therefore, evaluation of safety of the fibers needs to be conducted in both *in vivo* and *in vitro* studies using an inert fibers as negative controls. Additionally, evaluation of safety of well characterized fibers *in vitro* must be conducted in biologically feasible concentrations, sizes and numbers of fibers for used in *in vivo* experiments.

The choice of pulmonary cell type and source of cells; i.e. cell line, primary cells, is an important issue for *in vitro* experiments. Thus far most *in vitro* assays have utilized monolayer cells. Macrophages were used for evaluation of toxicity and cytokines production. Rodent fibroblasts were used for evaluation of toxicity and morphologic transformation after incubation with fibers. Human mesothelial cells *in vitro* are 100- and 1000-fold more sensitive to the cytotoxic effects of asbestos than human tracheobronchial epithelial cells or lung fibroblasts, the least sensitive cell type⁸³). Brody *et al.*⁸⁴) applied long-term culture of alveolar epithelial cells for up to 8 weeks, and seems to be possible macrophages and fibroblasts in co-culture with epithelial cells during long term experimental period.

Recently, reports on safety evaluation of MMMF *in vitro* studies are increasing. These findings have not been sufficient to confirm their toxicity and safety to date. Further and

more detail studies will be required using newer and sensitive techniques; e.g. molecular biology. The results from such studies *in vitro* should correlate with those of *in vivo* studies.

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