Original

Lung Injury and Oxidative DNA Damage Caused by the Amorphous Material Synthesized by Heating Chrysotile

Ayako Takata[1], Hiroshi Yamauchi[2], Tadao Toya[3], Masahito Aminaka[1], Yasushi Shinohara[3], Norihiko Kohyama[4] and Katsumi Yoshida[1]

[1]Department of Preventive Medicine, St. Marianna University School of Medicine

[2]Department of Public Health, School of Allied Health Science, Kitasato University

[3]National Institute of Occupational Safety and Health

[4]Natural Science Laboratory, Faculty of Economics, Toyo University

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Abstract

The conversion of chrysotile (CH) to forsterite (FO) by heat treatment is considered to be an acceptable technique for detoxifying chrysotile. From the perspectives of environmental burden and economic efficiency, however, it is desirable to maintain the heating temperature at which CH is detoxified as low as possible. The process of converting CH into FO generates an amorphous by-product, the biological effects of which have raised concerns. In this study, the biological effects of CH and an amorphous substance (CH660) that is synthesized by heating CH at 660℃ were compared with respect oxidative DNA damage and lung injury. Following the administration of a single 1-mg intratracheal dose of CH or CH660, we evaluated histopathological changes in the lung tissue, the lung concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG), and the localization of 8-OHdG for 540 days. For the control group, we administered a single intratracheal dose of sterile saline. In the early period following the intratracheal administration of CH, expression of 8-OHdG was observed in the bronchiolar and alveolar epithelial cells as well as in infiltrating inflammatory cells and granulomas; at the same time, an increase in the lung concentration of 8-OHdG was observed. As fibrosis progressed, expression of 8-OHdG became more apparent in the airway epithelial and inflammatory cells surrounding the fibrotic lesions; even on day 540, the expression of 8-OHdG was significantly higher than that of the control group ($p<0.001$). On the other hand, acute inflammatory responses were observed 3 days after administering an intratracheal dose of CH660. Expression of 8-OHdG was also observed in the bronchiolar epithelial and inflammatory cells, and the lung concentration of 8-OHdG was significantly higher than that of the control group ($p<0.001$). However, compared to the CH group, recovery from the inflammation caused by CH660 was more rapid, and expression of 8-OHdG was also rapidly diminished. On day 540, there was neither an occurrence of fibrosis in the lungs nor a significant increase in the concentration of 8-OHdG. This study clearly demonstrates that the amorphous material synthesized by heating CH has also acute effects associated with oxidative DNA damage and lung injury.

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—Key words—

chrysotile, detoxification, oxidative DNA damage

Introduction

Asbestos exposure causes asbestosis, lung cancer, mesothelioma, and pleural disease. In Japan, chrysotile [CH; $Mg_3Si_2O_5(OH)_4$] has been widely used as a building material. Accordingly, even though the use of asbestos is now prohibited, it has been predicted that over the next 20 years more than one million tons of debris con-

taining chrysotile will be generated every year by the demolition of buildings. This has prompted discussion on the need to both convert the asbestos-containing wastes into harmless and recyclable materials. Following amendment of the Waste Management and Public Cleansing Law, the "Accreditation program for detoxification processes" was established in order to regulate asbestos-containing wastes. In this context, many detoxification procedures other than the traditionally used melting treatment at temperatures higher than 1,500℃ have been studied and tested.

One potential strategy capitalizes on the fact that CH exhibits dehydroxylation at approximately 550–700℃, followed by recrystallization to form forsterite (FO; Mg_2SiO_4) at approximately 700–800℃[1)~3)]. When it is further heated at 1,000℃ to 1,200℃, a portion of the FO is converted into enstatite ($MgSiO_3$)[1)~3)]. Pilot testing has indicated that FO synthesized by heating CH at 1,000℃ can provide both refractoriness and reinforcement to materials, and it is thus a potentially attractive candidate for an alternative to asbestos in refractory products.

Most authors consider that the biological effects of the FO synthesized by heating CH are milder than those of CH. Cytotoxicity studies *in vitro* have reported that FO is less toxic than CH[1)4)5)]. Similarly, the intrapleural administration of FO to rats did not cause pleural neoplasm[5)]. Further, when the material produced by heating CH at 850℃ was intraperitoneally administered to rats, the incidence rate of peritoneal mesothelioma was reduced to half that induced by the administration of CH[6)].

In contrast, the biological effects of synthesized amorphous substance are reported to be severer than those of FO. *In vitro* studies indicated that the hemolytic activity and cytotoxicity of the amorphous substance are prominent when it is synthesized by heating CH at temperatures between 500 and 700℃—temperatures at which the crystal structure of CH is destroyed by dehydration of the water of crystallization and rearrangement occurs to form the FO structure[1)4)]. Furthermore, when an amorphous substance synthesized by heating CH at 600℃ was intraperitoneally administered to rats, severe neurotoxicity including death was observed[7)]. To date, however, there have been insufficient studies regarding the intratracheal administration of these amorphous substances; consequently, further detailed studies are required.

Reactive oxygen and nitrogen species appear to play an important role in the pathogenesis of asbestos-induced diseases. Although the mechanism by which asbestos exposure causes oxidative DNA damage has not been clarified, the direct involvement of free radicals generated by asbestos fibers and the indirect involvement of inflammation caused by the impaired macrophage phagocytosis have been suggested[8)9)].

Recently, 8-hydroxy-2'-deoxyguanosine (8-OHdG) has gained acceptance as a biomarker for oxidative DNA damage[10)~13)]. In workers who have been exposed to asbestos, significantly increased concentrations of 8-OHdG are observed in leukocyte DNA[14)~16)] and in urine[17)].

In the future, a massive amount of CH-containing waste will require processing; thus, biosafety should be taken into consideration when deciding the appropriate processing conditions for the detoxification of CH. In view of these considerations, we assessed the biological effects of the synthetic amorphous substance based on observations of oxidative DNA damage and histopathological changes in the lung tissue after administering a single intratracheal dose of CH or synthetic amorphous substance.

Materials and Methods

Materials

Fibers of less than 250 μm in length were separated from CH (grade AX) produced by Cassiar Mining Corp., British Columbia, Canada. This CH sample contained 2% Fe_2O_3 (ferric oxide) and 1% Al_2O_3 (aluminum oxide) as confirmed by X-ray fluorescence analysis. Measurements made using a scanning electron microscope (SEM) (S4700; Hitachi High-Technologies Corporation, Tokyo, Japan) indicated that fibers with an aspect ratio of 3 or greater had a geometric mean length of 2.26 μm (geometric standard deviation = 2.37) and a geometric mean diameter of 0.12 μm (geometric standard deviation = 1.53) .

The processing conditions required to synthesize an amorphous substance from CH were determined based on the data obtained from thermogravimetry-differential thermal analysis (TG-DTA) and X-ray diffraction analysis. The processing temperature was set to 660℃—a temperature at which the X-ray diffraction

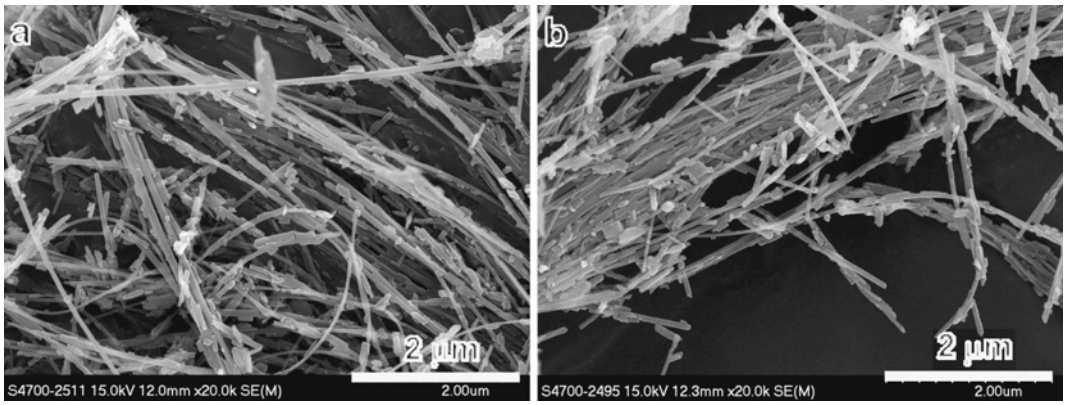

Fig. 1 SEM images of CH and CH660.
Magnification ×20,000.
(a) CH images. (b) CH660 images.

peak attributed to chrysotile diminishes within the temperature range at which CH loses water of crystallization.

In order to synthesize the amorphous substance (CH660), CH was heat-treated at 660℃ in an electric furnace for 1.5 hours. X-ray diffraction analysis revealed that the sample was composed mostly of amorphous substance. X-ray fluorescence analysis revealed that this CH660 sample contained 2% Fe_2O_3 and 1% Al_2O_3, which is roughly consistent with the CH sample. Although the CH660 sample contained fibers similar to CH, the long CH fibers were disrupted and the diameter of the CH600 fibers became thicker than that of the CH fibers (Fig. 1). SEM measurements indicated a geometric mean length of 1.94 μm (geometric standard deviation = 1.81) and a geometric mean diameter of 0.38 μm (geometric standard deviation = 1.74) .

Animal treatment

Each animal group consisted of 5 male Wistar rats (Clea Japan Inc., Tokyo, Japan). Rats were housed in a barrier facility with a 12 h/12 h light-dark cycle, in which the room temperature and humidity were maintained at 24 ± 1℃ and 55 ± 5%, respectively. There was a two-week acclimation period to the housing facility prior to experimentation.

CH or CH660 was administered to each rat at 10 weeks of age. One group received only CH and another other group received only CH660. Doses were prepared by suspending CH or CH660 in sterile saline solution using an ultrasonic cleaner (1 mg/0.5 ml). A single injection was administered intratracheally under halothane inhalation anesthesia. A third control group was administered 0.5 ml of sterile saline solution using the same method. Rats were dissected under deep anesthesia induced by intraperitoneal administration of pentobarbital sodium on days 3, 7, 14, 30, 90, 180, 360, and 540. All procedures were approved by the Animal Research Committee at the National Institute of Occupational and Safety Health, Japan.

Histopathological examination

Tissue samples were perfusion-fixed with phosphate-buffered 10% formalin. The lung and other major organ tissues were embedded in paraffin, sectioned, and then stained with Hematoxylin-eosin (HE) and Masson's trichrome stain for collagen deposition. Pulmonary histopathology injury was classified using the scale described by Toya et al.[18]. Histopathological examinations in the lungs focused on the following features: alveolar macrophage recruitment, inflammation, granuloma, epithelial hyperplasia, interstitial fibrosis, and neoplasm. Histopathological features in the lungs were evaluated by scoring the distribution and severity of each lung lesion on a scale of 0 to 3, with Grade 0 indicating "no changes or similar changes to those observed in the control group," Grade 1 indicating "focal and mild changes," Grade 2 indicating "moderate changes," and Grade 3 indicating "marked changes."

Immunohistochemical staining for 8-OHdG

Immunohistochemical staining for 8-OHdG in the lungs was performed using the same paraffin blocks of lung tissues as used in the histopathological examinations. After deparaffinization, the sections were auto-

Table 1 Lung histopathology severity for day 3 to day 540.

	Days after intratracheal administration																
	Control	CH								CH660							
	3–540	3	7	14	30	90	180	360	540	3	7	14	30	90	180	360	540
Bronchi and Bronchioles																	
Inflammation	0	2	2	2	1	1	1	1	1	1–2	1	0–1	0	0	0	0	0
Granuloma	0	2–3	3	2	2	2–1	2–1	1	1	0–1	0–1	0	0	0	0	0	0
Fibrosis	0	0	0	1	1–2	2	2	2	2	0	0	0	0	0	0	0	0
Reactive epithelial hyperplasia	0	2	2	2	2	1	1	1	1	1–2	1–2	1–2	1	0–1	0–1	0	0
Alveolar ducts																	
Inflammation	0	2–3	2–3	2	2–1	1	1	1	1	1	0–1	0–1	0–1	0–1	0	0	0
Granuloma	0	3	3	2	2–1	2–1	1	1	1	1	0–1	0–1	0	0	0	0	0
Fibrosis	0	0	0	1	1–2	2	2–3	3	3	0	0	0	0	0	0	0	0
Alveoli																	
Accumulation of PAM	0	2	2	2	2	1	1	1	1	1–2	1	0–1	0–1	0–1	0–1	0–1	0–1
Inflammation	0	2–3	2–3	1	1	1	1	1	1	1	0–1	0–1	0–1	0–1	0–1	0–1	0–1
Granuloma	0	1	1–2	1–2	1–2	1	1	0	0	0–1	0–1	0	0	0	0	0	0
Fibrosis	0	0	0	0	0–1	1	2	2–3	2–3	0	0	0	0	0	0	0	0

The severity of histological changes was scored as "mild," "moderate," and "marked" (grades 1–3, respectively). A score of "zero" indicates that the finding was not observed.
PAM; alveolar macrophages.

claved at 120℃ for 10 min for antigen retrieval. After quenching endogenous peroxidase activity, the sections were washed with phosphate-buffered saline (PBS), followed by skim milk for the reduction of nonspecific binding, and a final PBS wash. Anti-8-OHdG monoclonal antibody (MOG-020; Japan Institute for the Control of Aging, Shizuoka, Japan) was used as the primary antibody. After addition of anti-8-OHdG monoclonal antibody solution (dilution 1: 100), the slides were incubated overnight at 4℃. The slides were then washed with PBS and incubated with biotin-labeled secondary antibody (Dako Japan Inc., Tokyo, Japan) at room temperature. After a further wash, the slides were treated with StreptAB complex/HRP (Dako Japan Inc., Tokyo, Japan). The slides were washed again and treated with 3,3'-diaminobenzidine-tetrahydrochloride (Dako Japan Inc., Tokyo, Japan) in order to visualize the reactions. The nuclei were counterstained with Meyer's hematoxylin.

HPLC-electrochemical detection (ECD) of 8-OHdG in lung tissue

Approximately 0.1 g of lung sample was homogenized in 3 ml of ice-cold 0.15 *M* NaCl/0.1 *M* EDTA (pH 8.0) using a homogenizer (KINEMATICA, Switzerland). The nuclear fraction was collected via centrifugation at 1,500 × *g* for 15 min at 4℃. This fraction was stirred with 1.5 ml 0.15 *M* NaCl/0.1 *M* EDTA (pH 8.0). After the subsequent addition of 2 ml lysis buffer (Applied Biosystems, U.S.A) and 500 μl proteinase K (400 μg/ml; Wako Pure Chemical Industries, Ltd., Osaka Japan), the reaction mixture was incubated at 50℃ for 30 min. The reaction mixture was then mixed with 0.4 ml 2 *M* sodium acetate buffer (pH 4.5) and 8 ml ethanol, and DNA was collected using a Pasteur pipette. The DNA sample was cooled on ice and mixed with 100 μl of 100 U/ml nuclease P_1 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 5 μl of 2 *M* sodium acetate buffer (pH 4.5), and then incubated at 37℃ for 60 min. The DNA mixture was then mixed with 80 μl of 1 *M* Tris-HCl buffer (pH 7.5) and 20 μl of alkaline phosphatase (SIGMA, U.S.A.), and incubated again at 37℃ for 60 min. The sample was centrifuged at 10,000 × *g* for 3 min at 4℃, and the supernatant was collected and incubated at 50℃ for 60 min. After the incubation, the supernatant was subjected to centrifugal filtration using 0.22-mm Ultrafree MC filters (Millipore, U.S.A), followed by a further centrifugal filtration using Microcon Centrifugal Filter Units (Millipore, U.S.A) .

8-OHdG in the filtered sample was analyzed using HPLC-ECD detection. Measurements were made using a Jasco Gulliver Series HPLC system (JASCO Corporation, Tokyo, Japan) equipped with a Coulochem II electrochemical detector (ESA Inc., U.S.A.). At the same time, 2-deoxyguanosine (2-dG) was quantified using a UV detector. A reverse-phase partition column (Capcellpak C18 UG120, 4.6 × 250 mm; Shiseido, Tokyo, Japan) was used for HPLC. The column temperature and flow rate were 40℃ and 0.8 ml/min, respectively. For the mobile phase, an 8% methanol/10 mM NaH_2PO_4 solution was used. Standard solutions of 8-OHdG (Wako Pure Chemi-

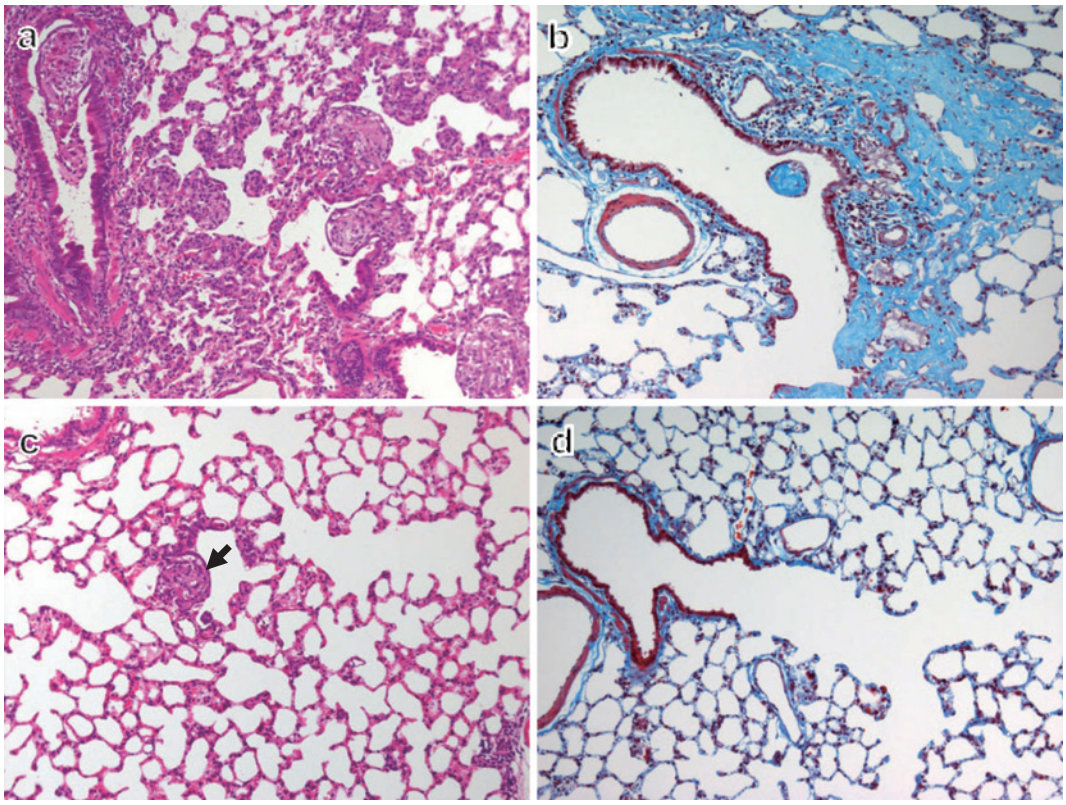

Fig. 2 Lung histopathology.
(a) CH day 3, H-E stain, magnification×200. Bronchiolitis and alveolitis were apparent. Foreign-body granulomas were apparent in the bronchioles and alveolar ducts.
(b) CH day 540, Masson's trichrome stain, magnification×200. Fibrotic lesions were apparent in the bronchioles and alveolar ducts, and extended into the surrounding alveoli.
(c) CH660 day 3, H-E stain, magnification×200. Mild infiltration of inflammatory cells was observed in the regions surrounding the bronchioles and in the alveoli. Microgranulomas were also observed (arrow).
(d) CH660 day 540, Masson's trichrome stain, magnification×200. Inflammation and microgranulomas were minimal, and no fibrotic or neoplastic lesions were apparent.

cal Industries, Ltd., Osaka, Japan) and 2-dG (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were diluted to 5–20 ng/ml and 1–5 μg/ml, respectively. The level of 8-OHdG in the DNA was expressed as the number of residues per 10^6 guanine.

Statistical analysis

Lung concentrations of 8-OHdG are summarized as mean ± standard deviation (SD). Data from the control group and the CH and CH660 groups, including lung concentrations of 8-OHdG, were subjected to one-way analysis of variance. Post-hoc pairwise differences were assessed using Tukey's honestly-significant difference test (SPSS 15.0-J; SPSS Japan Inc., Tokyo, Japan) .

Results

Histopathological examination

Pulmonary lesions observed during the observational period are summarized in Table 1. No histopathological changes were observed from day 3 until day 540 in the control group, including inflammatory, granulomatous, and fibrotic lesions.

Significantly more inflammatory responses were observed in the bronchioles, alveolar ducts, and alveoli on day 3 for the CH group compared to controls. In particular, granulomas were identified in the bronchioles and alveolar ducts (Fig. 2a). Bronchiolar epithelial hyperplasia and goblet cell metaplasia were also apparent.

Inflammatory responses gradually diminished after day 14 for the CH group. After day 30, collagen deposition within granulomas became more apparent in bronchioles and alveolar ducts. Interstitial fibrosis was

more prevalent than in controls on day 540, and was intense and extended to the bronchioles, alveolar ducts, and alveoli (Fig. 2b). Inflammatory cells infiltrated locally in the regions surrounding the granuloma and fibrotic lesions.

On the other hand, lung lesions in the CH660 group were more transient compared with those observed in the CH group. Inflammatory responses were observed in the bronchioles, alveolar ducts, and alveoli on day3. Furthermore, microgranulomas were apparent in the alveolar ducts and alveoli (Fig. 2c). However, these inflammatory responses were milder than those of the CH group. Inflammatory responses had subsided by day 14. Neither the inflammatory responses nor the progressive fibrosing lesions detected in the CH group were apparent in the CH660 group on day 540 (Fig. 2d). No neoplastic lesions were found in the CH and CH660 groups.

Immunostaining for localization of 8-OHdG in lung tissue.

No 8-OHdG overexpression was detected via immunostaining for the control group during the entire observation period (Fig. 3a, b). In contrast, 8-OHdG expression in the CH group was apparent starting on day 3 in macrophages, inflammatory cells that had infiltrated into the bronchioles, alveolar ducts, and alveoli. Nuclei of inflammatory cells within granulomas were also positive for 8-OHdG. Expression of 8-OHdG was not restricted to inflammatory cells, but extended into bronchiolar and alveolar epithelial cells. Expression of 8-OHdG was more marked on days 7 and 14 (Fig. 3c), and became locally-intensified in bronchiolar and alveolar epithelial cells and inflammatory cells surrounding fibrotic lesions. This expression pattern persisted until day 540 (Fig. 3 d) .

The expression of 8-OHdG in the CH660 group was more transient compared to the CH group. Expression of 8-OHdG was observed in the bronchiolar and alveolar epithelial cells as well as in the infiltrated inflammatory cells (Fig. 3e). Expression of 8-OHdG was also apparent in the microgranulomas. After day 14, however, the expression of 8-OHdG decreased gradually. Expression was not detected on day 540 (Fig. 3f) .

Lung tissue concentration of 8-OHdG

Changes in 8-OHdG concentration in lung tissue during the observation period are summarized in Fig. 4. The lung concentration of 8-OHdG on day 3 for the CH660 group was $19.6 \pm 0.6/10^6$dG, which was significantly higher than that of the control group ($2.51 \pm 1.09/10^6$dG) , ($p < 0.001$). Thereafter, lung concentrations of 8-OHdG decreased rapidly. Differences between the CH660 group and controls were slight and not statistically significant between day 7 and day 540.

In contrast, lung concentrations of 8-OHdG were significantly higher for the CH group compared to controls for the entire period, and the difference was most marked ($44.7 \pm 12.2/10^6$dG) on day 14 ($p < 0.001$). Dynamics of lung 8-OHdG concentration were thus markedly different for the CH and CH660 groups.

Discussion

The purpose of this study was to assess the biological effects of an amorphous by-product potentially produced under conditions of insufficient heating during the synthesis of FO from CH. To this end, we used an amorphous substance (CH660) produced by heating CH at 660℃, the processing conditions of which were determined based on TG-DTA data. For 540 days following the administration of a single intratracheal dose of CH or CH660 to rats, we evaluated the resultant oxidative DNA damage and lung injuries.

Immunostaining revealed localized expression of 8-OHdG in macrophages, infiltrating inflammatory cells, and granulomas starting three days after CH administration. This expression was not restricted to inflammatory cells, but extended to bronchiolar and alveolar epithelial cells. These observations are consistent with results of an examination of 8-OHdG concentrations made using the HPLC-ECD method (Fig. 4). Notably, persistent expression of 8-OHdG was apparent 540 days after administration using both instrumental analysis of 8-OHdG and immunostaining—a pattern indicative of the progression of fibrosis. This finding is consistent with the hypothesis that overexpression of 8-OHdG is linked to the excessive production of reactive oxygen species that is mediated by inflammatory responses induced by incomplete phagocytosis of macrophages[8)9)].

On the other hand, results of pulmonary inflammation and oxidative DNA damage in the CH660 group indicate that CH660 causes only acute effects in the lung and that, unlike CH, it does not cause chronic effects;

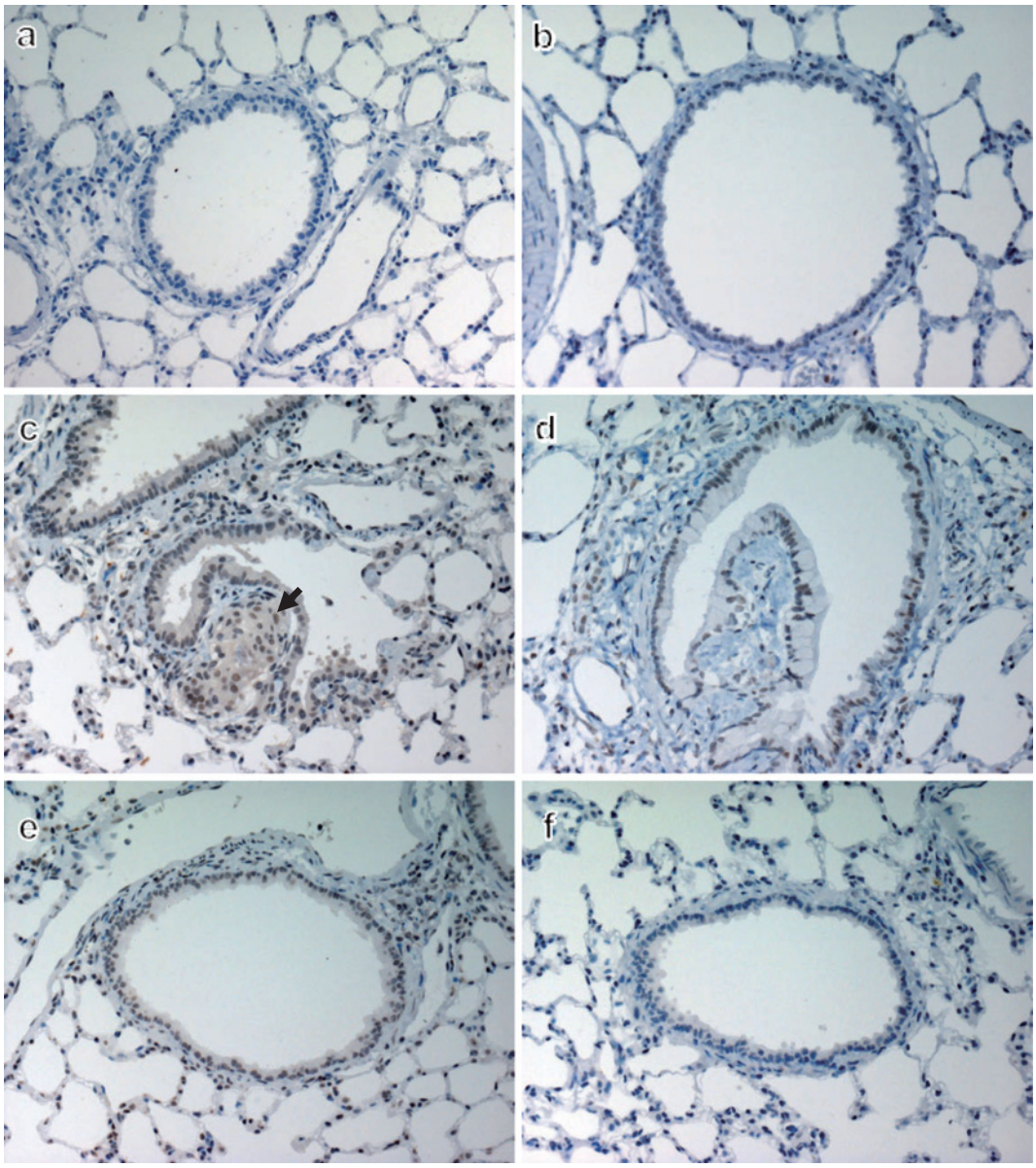

Fig. 3 Immunohistochemical analysis of 8-OHdG in the lungs.
Anti-8-OHdG monoclonal antibody was used for immunostaining.
(a) Control day 14, magnification × 400. No 8-OHdG expression was apparent in airway epithelial and inflammatory cells.
(b) Control day 540, magnification × 400. No 8-OHdG expression was apparent in airway epithelial and inflammatory cells.
(c) CH day 14, magnification × 400. Nuclei of bronchiolar and alveolar epithelial cells as well as those of inflammatory cells (neutrophils, eosinophils, and macrophages) were positive for 8-OHdG. Nuclei of inflammatory cells within granulomas were also positive for 8-OHdG (arrow).
(d) CH day 540, original magnification × 400. Nuclei of bronchiolar epithelial cells and interstitial inflammatory cells were positive for 8-OHdG.
(e) CH660 day 3, 8-OHdG, magnification × 400. Expression of 8-OHdG was observed in the airway epithelial and inflammatory cells.
(f) CH660 day 540, 8-OHdG, magnification × 400. No expression of 8-OHdG was observed in the airway epithelial and inflammatory cells.

consequently, the biological effects of CH660 are considered to be less severe than those of CH.

The cytotoxic effects of the amorphous substance on rat macrophages are reported to be strongest when it is synthesized by heating CH at 650℃ [1]. In the present study, at 3 days after the intratracheal administration of CH660, expression of 8-OHdG was observed in the infiltrated alveolar macrophages and neutrophils as well

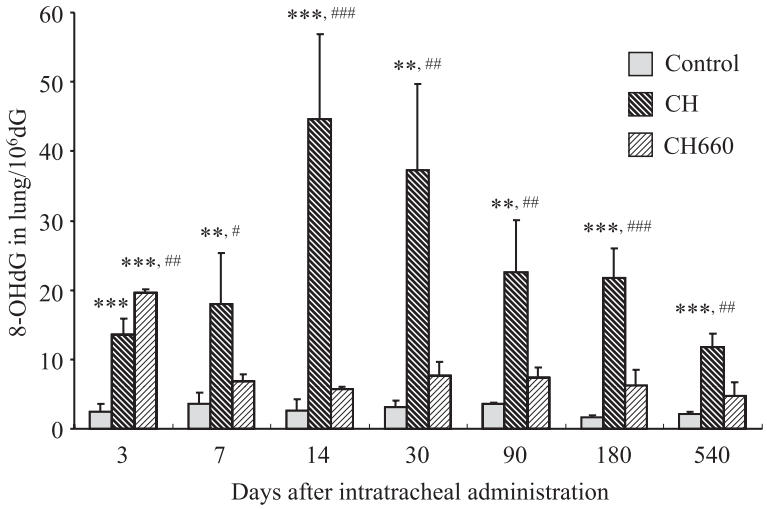

Fig. 4 Time course of changes in lung 8-OHdG concentrations.
Data are presented as mean ± SD. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (compared to the control group). $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ (compared to the CH group).

as in the bronchiolar and alveolar epithelial cells. Thus, it is suggested that inflammation was induced by the cytotoxicity of CH660 to macrophages, resulting in the increased expression of 8-OHdG.

However, recovery from the oxidative DNA damage and inflammation caused by CH660 was more rapid than recovery from the injuries caused by CH. The biological effects of asbestos and similar fibrous materials are influenced by the solubility and length of the fibers, and particularly by the lung biopersistence of fibers exceeding 20 μm in length that cannot be completely phagocytized by macrophages[19]. Since the CH660 sample is composed mostly of amorphous substance, its solubility is probably higher than the crystalline fibers that characterize CH; thus, the lung persistence of CH660 is predicted to be low. At 1 year after an intratracheal administration of CH, there was no decrease in the number of fibers exceeding 5 μm. In contrast, 30 days after an intratracheal administration of CH660, the number of fibers of various lengths decreased; the number of fibers exceeding 20 μm, in particular, was greatly decreased to one-fifth the initial level (unpublished data). Previously, severe neurotoxicity was observed following the intraperitoneal administration of amorphous substances that were synthesized from CH, and this severe neurotoxicity was suggested to be linked to the solubility of the amorphous substances[7].

This study clearly demonstrates that the amorphous material synthesized by heating CH has also acute effects associated with oxidative DNA damage and lung injury. However, there are inconsistencies regarding data on the biological effects obtained from intratracheal administration studies and those obtained from intraperitoneal administration studies; thus, further studies are required in this field of research.

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Reprint request:
Ayako Takata M.D, Ph.D.
Department of Preventive Medicine, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, 216-8511, Japan.

別刷請求先 〒216–8511 川崎市宮前区菅生 2—16—1
聖マリアンナ医科大学予防医学教室
高田 礼子

クリソタイルを焼成処理し合成した非晶質材料の肺傷害と酸化的 DNA 損傷

高田　礼子[1)]，山内　　博[2)]，戸谷　忠雄[3)]，網中　雅仁[1)]
篠原也寸志[3)]，神山　宣彦[4)]，吉田　勝美[1)]

[1)]聖マリアンナ医科大学予防医学
[2)]北里大学医療衛生学部公衆衛生学
[3)]独立行政法人労働安全衛生総合研究所
[4)]東洋大学経済学部自然科学研究室

—キーワード—
クリソタイル，無害化，酸化的 DNA 損傷

クリソタイル（chrysotile；CH）の無害化処理として，加熱によりフォーステライト（forsterite；FO）に変換する技術がある．環境負荷，経済性の観点から，CH をより低温で焼成し無害化することが期待されている．CH の FO への変換過程で生成される非晶質材料は，生体影響が危惧されている．本研究では，CH を 660℃ で焼成した非晶質試料（CH660）について，肺傷害と酸化的 DNA 損傷を CH と比較し検討した．ラットに CH と CH660 をそれぞれ 1mg 一回気管内投与し，540 日後まで肺の病理組織学的変化，肺中 8-hydroxy-2'-deoxyguanosine（8-OHdG）の濃度と免疫染色による発現をそれぞれ評価した．CH の気管内投与後早期における 8-OHdG の発現は細気管支・肺胞上皮，炎症細胞，さらに肉芽腫において認められ，肺中 8-OHdG 濃度も上昇した．線維化の進展に伴い，病変周囲の気道上皮や炎症細胞での 8-OHdG 発現は顕著で，投与後 540 日でも対照群と比べて有意に高値を示した（$p<0.001$）．これに対して，CH660 の気管内投与 3 日後，急性炎症に伴い 8-OHdG は細気管支上皮や炎症細胞において発現がみられ，肺中 8-OHdG 濃度も対照群と比べて有意に上昇した（$p<0.001$）．しかし，炎症の回復は CH に比べて速く，8-OHdG の発現も急速に減少した．投与後 540 日において，肺の線維化は生じず，8-OHdG 濃度も有意な上昇はみられなかった．この研究から，CH から合成された非晶質材料の肺傷害と酸化的 DNA 損傷に関して，とくに投与初期に CH と類似の急性影響が生じることを確認した．

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