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RAT MODEL DEMONSTRATES A HIGH RISK OF TREMOLITE BUT A LOW RISK OF ANTHOPHYLLITE FOR MESOTHELIAL CARCINOGENESIS

DILINUER AIERKEN[1], YASUMASA OKAZAKI[1], SHAN HWU CHEW[1], AKIHIRO SAKAI[1], YUE WANG[1], HIROTAKA NAGAI[1], NOBUAKI MISAWA[1], NORIHIKO KOHYAMA[2], and SHINYA TOYOKUNI[1]

[1]*Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*National Science Laboratory, Faculty of Economics, Toyo University, 5-28-20, Tokyo, Japan*

ABSTRACT

Asbestos was abundantly used in industry during the last century. Currently, asbestos confers a heavy social burden due to an increasing number of patients with malignant mesothelioma (MM), which develops after a long incubation period. Many studies have been conducted on the effects of the asbestos types that were most commonly used for commercial applications. However, there are few studies describing the effects of the less common types, or minor asbestos. We performed a rat carcinogenesis study using Japanese tremolite and Afghan anthophyllite. Whereas more than 50% of tremolite fibers had a diameter of < 500 nm, only a small fraction of anthophyllite fibers had a diameter of < 500 nm. We intraperitoneally injected 1 or 10 mg of asbestos into F1 Fischer-344/Brown-Norway rats. In half of the animals, repeated intraperitoneal injections of nitrilotriacetate (NTA), an iron chelator to promote Fenton reaction, were performed to evaluate the potential involvement of iron overload. Tremolite induced MM with a high incidence (96% with 10 mg; 52% with 1 mg), and males were more susceptible than females. Histology was confirmed using immunohistochemistry, and most MMs were characterized as the sarcomatoid or biphasic subtype. Unexpectedly NTA showed an inhibitory effect in females. In contrast, anthophyllite induced no MM after an observation period of 550 days. The results suggest that the carcinogenicity of anthophyllite is weaker than formerly reported, whereas that of tremolite is as potent as major asbestos as compared with our previous data.

Key Words: asbestos, mesothelioma, tremolite, anthophyllite

INTRODUCTION

Asbestos is a fibrous silicate mineral. Because of its cost-effective heat-, acid- and friction-resistance, substantial quantities of asbestos were used in the last century for various industrial purposes, including in construction materials, clothing and components for machine parts. Epidemiologists observed the association between asbestos exposure and malignant mesothelioma (MM) in the 1960s[1)], and the International Agency for Research on Cancer (IARC) declared in 1987 that all asbestos is a definite carcinogen for humans[2)]. At present, MM induced by asbestos

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Corresponding author: Shinya Toyokuni, MD, PhD; Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
Tel: +81-52-744-2086; Fax: +81-52-744-2091; E-mail: toyokuni@med.nagoya-u.ac.jp

is an ongoing, worldwide social problem because of the disease's lethality and the associated medical costs. Numerous court cases are underway between MM patients and asbestos companies or governments. Because of the extremely long incubation period, which can be 30–40 years after exposure, the peak MM incidence in Japan is expected to occur in 2025, and over 100,000 new patients are expected in the next 40 years[3]. The Japanese government banned all asbestos in 2006 after "Kubota shock"[4] and enacted a law providing all people suffering from pathologically diagnosed MM a financial allowance. However, asbestos is still produced and/or consumed in many developing countries, which will increase the incidence of MM in the near future, especially as treatment for major infectious diseases improves in those countries[5].

Chrysotile (white asbestos), crocidolite (blue asbestos) and amosite (brown asbestos) are all categorized as major asbestos. Substantial data are available on major asbestos fibers[2, 6], including studies published by the authors of this study[7-11]. Crocidolite has been used in most of the asbestos cell culture experiments. However, recent studies on minor asbestos, actinolite, tremolite and anthophyllite, are lacking. We performed a carcinogenesis experiment with Japanese tremolite ($[Ca_2Mg_5Si_8O_{22}(OH)_2]_n$) and Afghan anthophyllite ($[(Mg,Fe^{2+})_7Si_8O_{22}(OH)_2]_n$). The mesothelial carcinogenicity of tremolite with short fibers was potent, whereas Afghan anthophyllite did not induce MM within a period of 550 days.

MATERIALS AND METHODS

Materials

Japanese tremolite (Yamaga City, Kumamoto, Japan) of short fiber preparation and Afghan anthophyllite (36-mesh) were kind gifts from Dr. Nobuhiko Kohyama. The chemical composition of these two fiber types compared with other anthophyllite preparations is summarized in Table 1 and is based on previous analyses[12]. Characterization of the tremolite preparation used in this study has been previously published[13]. We dispersed asbestos fibers in saline at a concentration of 5 mg/ml, sonicated the solution vigorously immediately before use, and performed intraperitoneal injections in rats. We observed fiber preparations using a BZ-9000 microscope at 1,000x with oil immersion (Keyence, Osaka, Japan). Nitrilotriacetic acid disodium salt (nitrilotriacetate; NTA) was used as an iron chelating agent and was procured from Nacalai, Kyoto, Japan.

Animal experiments

The animal experiment committee of the Nagoya University Graduate School of Medicine approved the experiments performed in this study. Six-week-old 150 female and 120 male F1 Fischer-344 × Brown-Norway hybrid rats (Charles River, Japan) were used, which is consistent with our previous experiments. No spontaneous MM has been reported in these rats[9]. A total of 270 animals were used, and they were divided into the following three groups: untreated control (NTA or saline), tremolite and anthophyllite. We used two injection doses of each asbestos fiber (4 groups of 54 animals each, consisting of 24 males and 30 females). The animals received two intraperitoneal injections of either 0.5 or 5 mg asbestos fibers in 1 ml saline with a week-interval. The total asbestos injected was either 1 or 10 mg per animal. Half of each group received intraperitoneal injections of 80 mg/kg NTA once a week for 12 weeks to promote an iron-catalyzed Fenton reaction, as previously described[14, 15]. Either NTA alone of the same dose or physiological saline solution was administered to the remaining rats as two control groups. Animals were maintained in a specific pathogen-free environment at 24°C with a 12-h dark/light cycle. When the animals showed massive ascites or were dying, they were euthanized, and an autopsy examination was performed. The experiments were terminated 550

Table 1 Chemical composition of minor asbestos

	Anthophyllite			Tremolite
	UICC**	Afghan*	Matsubase, Kumamoto, Japan**	Yamaga, Kumamoto, Japan*
SiO_2	55.31	57.97	56.28	50
TiO_2	0.03	0.01	0.02	Trace
Al_2O_3	0.99	0.05	0.59	0.07
Fe_2O_3	(6.32)#	ND	3.60	(7.2)#
FeO	5.75	8.38	5.96	6.6
MnO	0.17	0.33	0.30	0.42
MgO	31.15	28.81	26.74	25
CaO	0.29	0.28	0.49	17
Na_2O	0.03	0.02	0.02	0.02
K_2O	0.43	0.00	0.02	0.01
$H_2O(+)$	4.22	2.64	4.50	
$H_2O(-)$	1.31	0.07	1.69	
Total(%)	99.68	98.56	100.21	99.12

*Used in the present study. **Used in previous studies (UICC[22]; Matsubase[23]). Trace, <0.01%; ND, not detected (<0.001%). #Fe_2O_3 is the result of oxidation with heating during the measurement process and is recalculated to FeO rather than included in the total. Refer to 12) for details. This table was modified from 29).

days after the first injection of asbestos. Excised organs were fixed in phosphate-buffered 10% formalin and underwent paraffin embedding and routine hematoxylin and eosin staining processes or immunohistochemistry. Additionally, Perls' iron staining and Masson trichrome staining were performed. Macroscopic grading of MM was performed as previously described[16]. In brief, the diameter of the largest nodule was classified as <1, 1–5 or ≥5 mm (1–3 points), dissemination was classified as <25, 25–50 or ≥50% of the abdominal cavity (1–3 points), and the obtained two points were added. The severity was then graded as low (2 and 3 points), moderate (4 points) or high (5 and 6 points).

Immunohistochemistry

For immunohistochemical analysis, immunoperoxidase methods were used as previously described using the following antibodies[17, 18].

Antibodies

Anti-podoplanin rabbit polyclonal antibody (KS-17) was from Sigma (St. Louis, MO). Anti-multi-cytokeratin mouse monoclonal antibody (RTU-AE1/AE3) was from Novocastra (Newcastle, UK). Anti-desmin mouse monoclonal antibody (clone D33) was from DAKO (Carpinteria, CA). Anti-calretinin rabbit monoclonal antibody (clone SP13) was from Abcam (Cambridge, MA). Anti-S-100 polyclonal antibody was from DAKO. Anti-C-ERC/mesothelin monoclonal antibody was from IBL (Takasaki, Gunma, Japan). Anti-WT1 rabbit polyclonal antibody (C-19) was from Santa Cruz (Santa Cruz, CA). Secondary antibody was Histofine Simplestain rat MAX-PO (Multi) from Nichirei Bioscience (Tokyo).

Statistical analysis

Kaplan-Meier and other statistical analyses were performed using GraphPad Prizm 5 software (Graphpad; San Diego, CA). P values for the Kaplan-Meier analysis were calculated using the log-rank test. Other analyses used were the unpaired *t*-test, modified for unequal variances when necessary; and the chi-square test. $P < 0.05$ was considered statistically significant.

RESULTS

Fiber morphology

The number of fibers per dry weight for the preparations used was 11.76×10^{10} fibers/g for tremolite and 2.81×10^{10} fibers/g for anthophyllite. There was a distinct difference in the fiber morphology of the two asbestos fibers. The diameter of more than 50% of tremolite fibers was < 500 nm, and the diameter was uniform throughout the entire fiber length (Fig. 1A and B). In contrast, the diameter of most anthophyllite fibers was > 1000 nm, and they sometimes had irregular ends, though the samples also contained thinner needle-like fibers. Some of the thin anthophyllite fibers revealed either one-sided bluntness or slight bulginess in the center (Fig. 1C-F). Approximately 15% of the tremolite fibers and 100% of the anthophyllite fibers were longer than 20 μm (Fig. 1G).

Tremolite induced malignant mesothelioma (MM) but anthophyllite did not

Tremolite induced peritoneal MM in a dose-dependent manner (Fig. 2). Most rats (96.1%; male NTA, 10/10; male saline, 11/11; female NTA, 13/15; female saline, 15/15; 3 males died during injection period) administered with 10 mg tremolite developed MM within 550 days of the initial asbestos administration, and 51.9% of rats administered with 1 mg tremolite developed MM within 550 days (male NTA 11/12; male saline 9/12; female NTA 5/15; female saline 3/15). Males were more susceptible than females (tremolite 1 mg; NTA, P=0.0031; saline, P=0.0007; NTA+saline combined, P<0.0001 with the log rank test; tumor incidence at day 550, $P=3.455 \times 10^{-5}$ with the chi-square test). Unexpectedly, repeated post-administration of NTA significantly delayed mortality only in the female 10 mg tremolite group (P=0.0072). In all the other groups, NTA had no significant effects. Surprisingly, rats that received injections of anthophyllite did not develop MM (Fig. 2).

Animals with MM (tremolite groups only) often suffered from massive bloody ascites. Macroscopically, the tumors were scattered in the peritoneal cavity, and the largest nodules were most commonly longer than 5 mm in diameter. These tumors sometimes proliferated on the liver surface (Fig. 3A and B). The severity of dissemination was different in each case; therefore, this was the factor used to determine the macroscopic grading of MM (Fig. 3B). The sarcomatoid subtype of MM was the most common histological finding (Fig. 3C). Pathologic diagnosis was confirmed using immunohistochemistry, which revealed positivity for cytokeratin (AE1/3), calretinin, podoplanin, mesothelin and WT1 in the epithelioid subtype and positivity for cytokeratin, mesothelin and WT1 in the sarcomatoid subtype. Desmin (a myogenic marker) and S-100 (a neural marker) were negative or only partially positive (Fig. 4A and 4B). We applied the same criteria used for human MM[19] for the pathologic diagnosis. We observed heavy and scattered iron deposits in the mesothelial cells and near the MM in most rats injected with tremolite (Fig. 5A-D). However, iron deposition was much less in rats injected with anthophyllite (Fig. 5E and F).

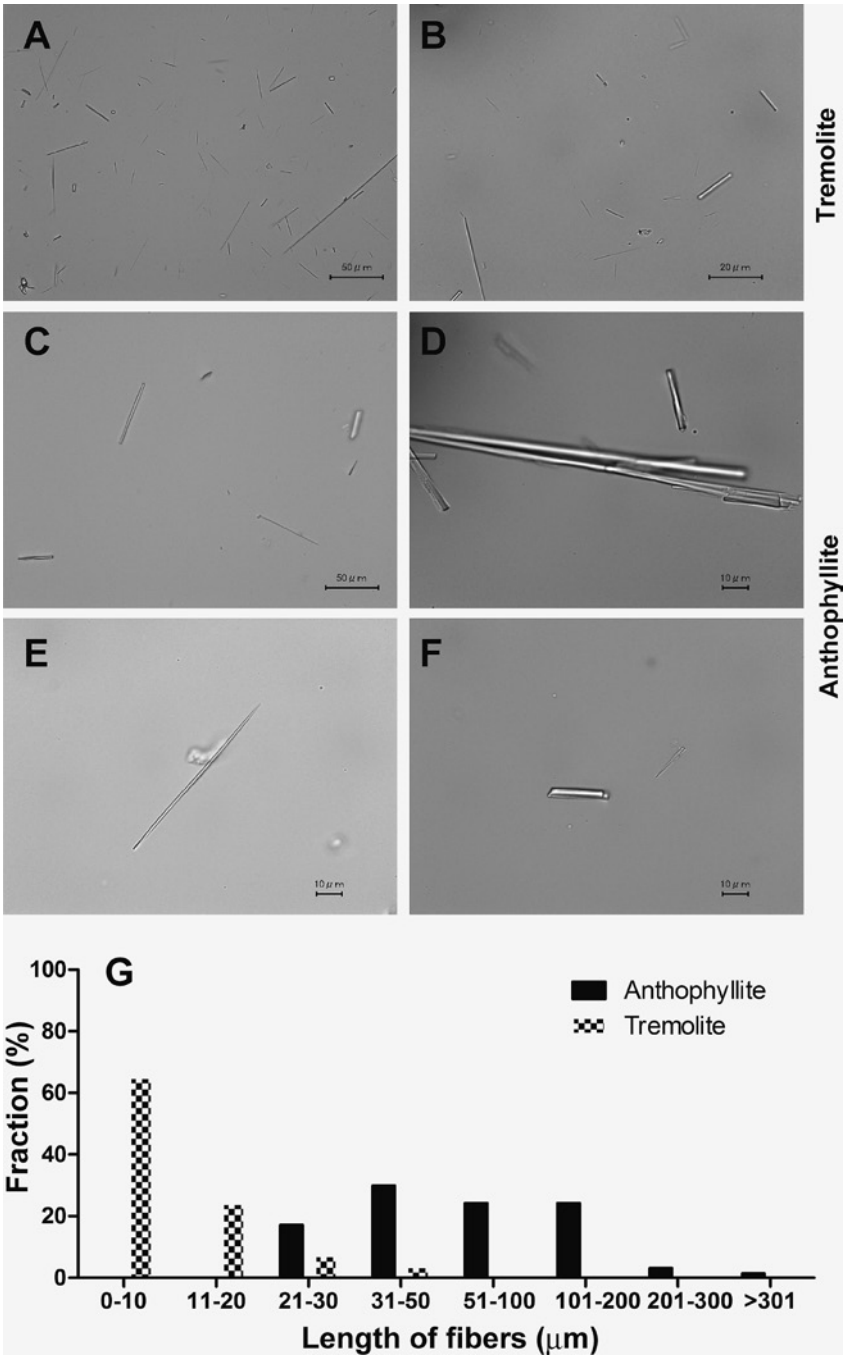

Fig. 1 Morphological analysis of the tremolite (A, B and G) and anthophyllite (C-G) fibers used in the present study. Anthophyllite is longer and larger in diameter than tremolite. Refer to text for details (bar = 50, 20 and 10 μm in AC, B and D-F, respectively).

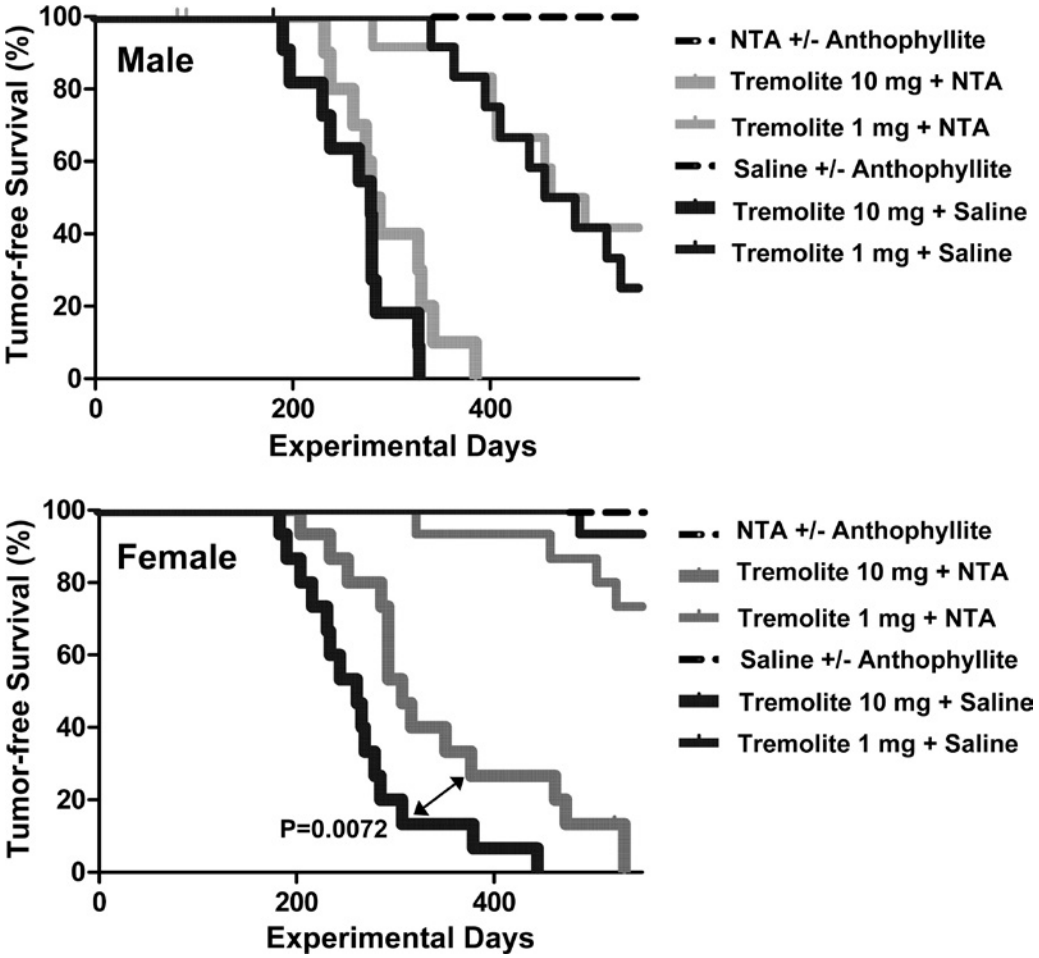

Fig. 2 Survival curve after intraperitoneal administration of minor asbestos. Lethal malignant mesothelioma (MM) was induced by tremolite in a dose-dependent manner but not by anthophyllite. Results from the 1 mg administration groups suggest males were more susceptible to developing MM (NTA+saline groups combined, $P<0.0001$ with the log rank test; tumor incidence at day 550, $P=3.455 \times 10^{-5}$ with the chi-square test). Refer to text for details. NTA, nitrilotriacetate.

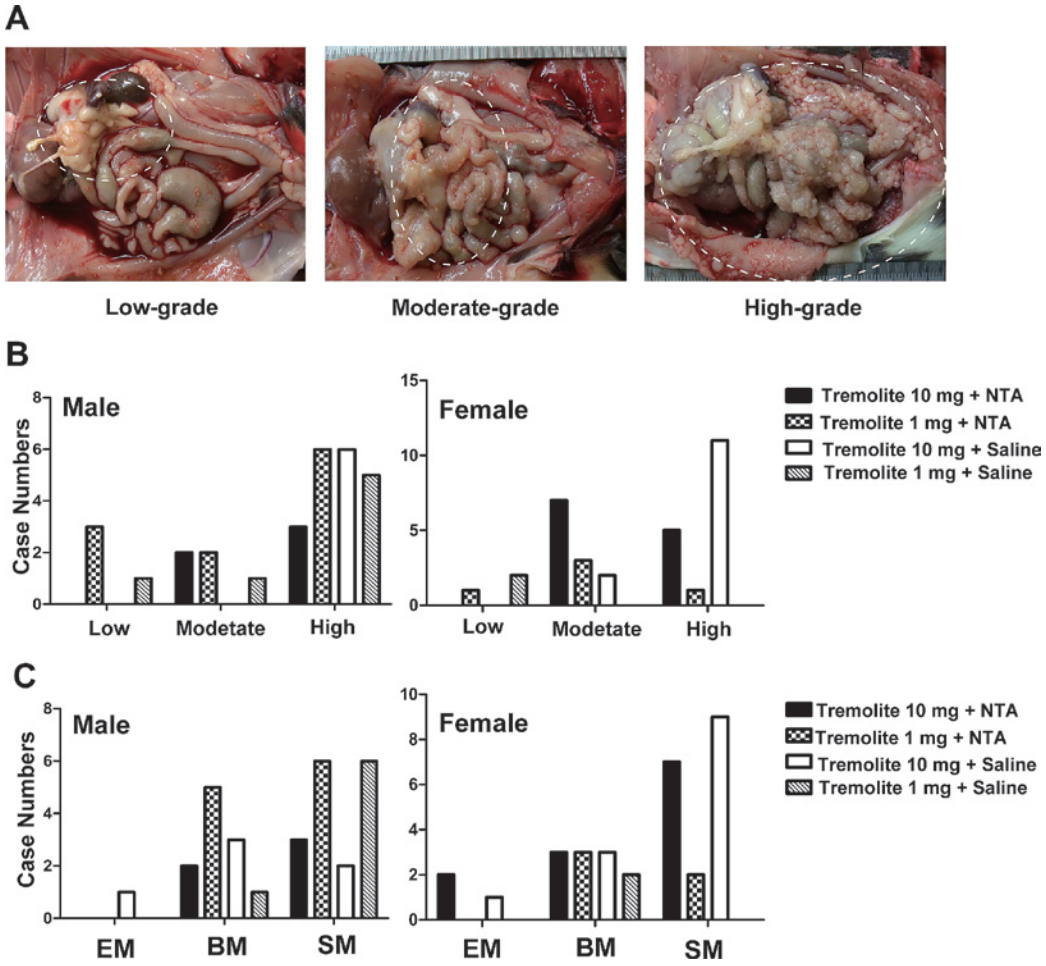

Fig. 3 Representative macroscopic appearance of the tremolite-induced peritoneal MM in rats, their invasive behavior and histological subtypes. A: Classification of invasive behavior. Interrupted circle indicates the location of MM. Refer to text for details. B: Invasive behavior of MM induced by tremolite. C: Classification of the histological subtypes. EM, epithelioid subtype; BM, biphasic subtype; SM, sarcomatoid subtype. Some data for B and C are lacking due to postmortem degeneration. MM, malignant mesothelioma; NTA, nitrilotriacetate.

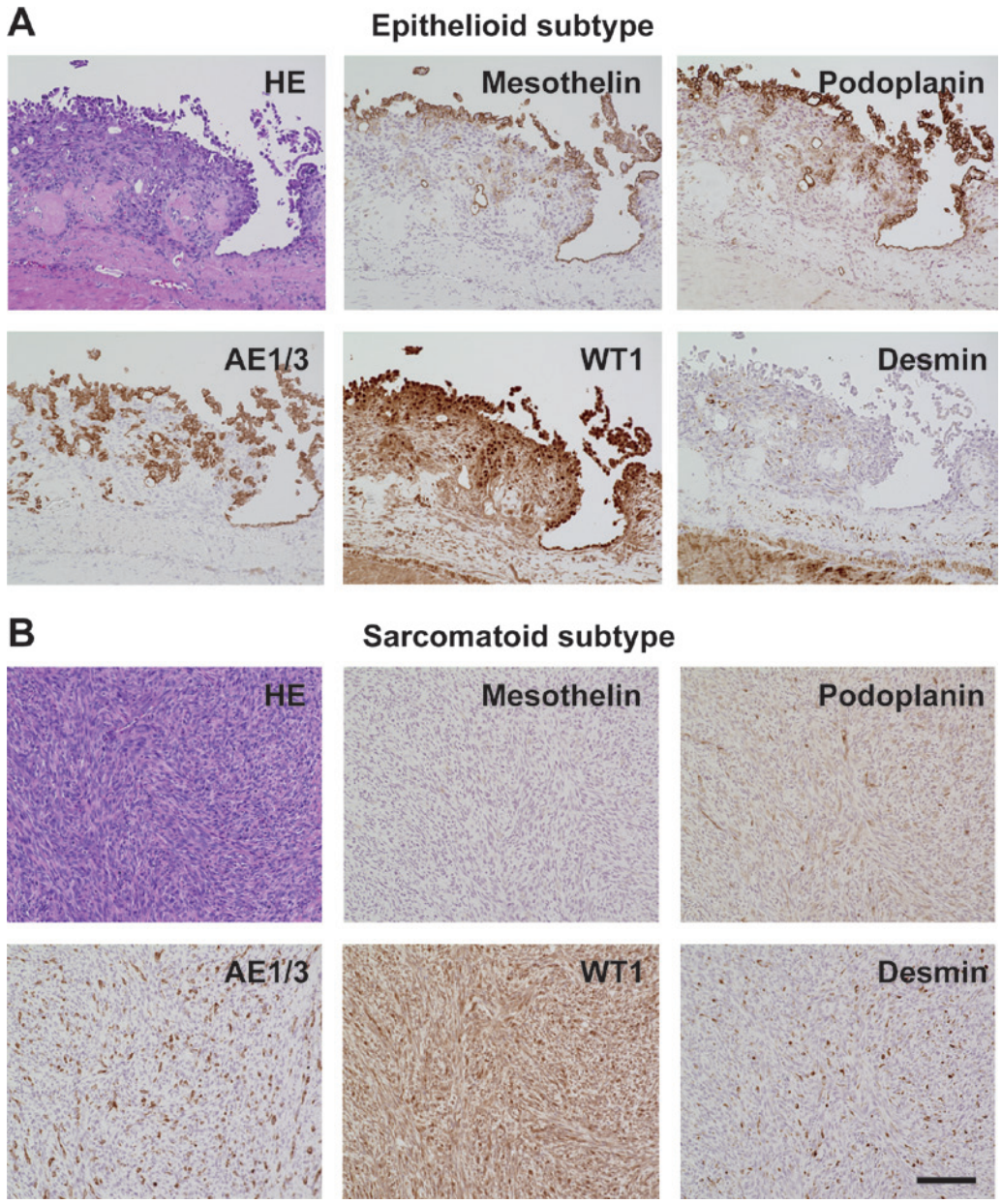

Fig. 4 Representative immunohistochemical analysis of tremolite-induced malignant mesothelioma. Refer to text for details. HE, hematoxykin and eosin; mesothelin and podoplanin, mesothelial marker; AE1/3, pan-cytokeratin; WT1, Wilms tumor 1 oncogene; desmin, myogenic marker (brown color as positivity; bar = 100 μm).

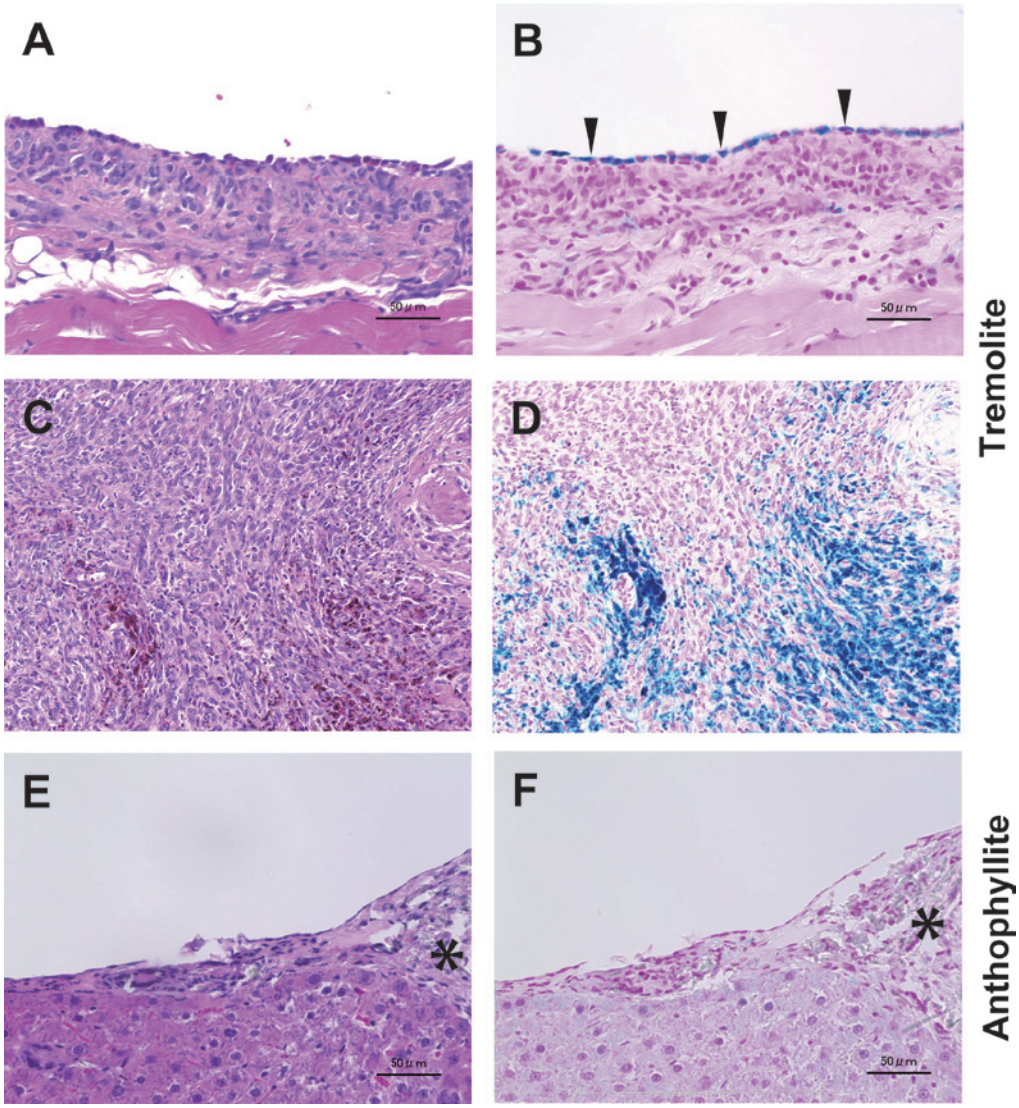

Fig. 5 Massive iron deposition in mesothelia during tremolite-induced mesothelial carcinogenesis and MM but not after anthophyllite administration. A: Mesothelia on diaphragm (HE). B: Mesothelia on diaphragm accumulate iron (arrow heads; Perls' iron staining; green color as positivity). C: Tremolite-induced MM, sarcomatoid subtype (HE). D: Surrounding tissue of tremolite-induced MM, sarcomatoid subtype, accumulate iron (Perls' iron staining). E: Liver surface after intraperitoneal anthophyllite administration. *, anthophyllite-induced granuloma. F: Mesothelia do not accumulate iron after anthophyllite administration. These are representative figures and these tissues were obtained at autopsy of rats more than 1 year after asbestos administration (bar = 50 μm). MM, malignant mesothelioma.

DISCUSSION

Fibrous minerals have been unexpected causes of human cancer. Few carcinogenesis studies have been performed on minor asbestos. We studied the carcinogenicity of two minor asbestos types, tremolite and anthophyllite, after characterization. A major fraction of each fiber presented

with a distinct morphology; namely, the tremolite fibers were short (length < 20 μm) and thin (diameter < 500 nm), and the anthophyllite fibers were long (length > 20 μm) and thick (diameter > 500 nm). The resolution limit of light microscopy is 200 nm, and we confirmed fiber diameters of less than 500 nm using a BZ9000 light microscope, which is less expensive and allows for immediate evaluation. Stanton's hypothesis is well known for fiber-induced carcinogenesis, which is directed to macrophages as "frustrated phagocytosis", and it hypothesizes that shorter fibers (< 250 nm) or longer fibers (> 20 μm) are more carcinogenic[20]. The results of our animal experiments suggest that the fiber diameter is a more critical factor than the fiber length in the asbestos-induced carcinogenic process by intraperitoneal administration. Recently, we found similar results using multiwalled carbon nanotubes, though the MM-susceptible diameter was different (50 nm)[21].

Tremolite from Yamaga City, Kumamoto, Japan, was potently carcinogenic after intraperitoneal injection in rats and generated MM that was confirmed using immunohistochemistry. The carcinogenic potential of tremolite, tested within the period required for mesothelial carcinogenesis, was stronger than that of the chrysotile, crocidolite and amosite asbestos types[9] and was as potent as multiwalled carbon nanotubes with a 50 nm-diameter[21] with experiments using the same F1 rats in our laboratory. We assume that it is not coincidence that of the rats with MM induced by tremolite or multiwalled carbon nanotubes, the majority developed the more malignant sarcomatoid subtype. It is still unknown at present which factors are responsible for differentiating between the epithelioid and sarcomatoid MM subtypes. There two hypotheses for this are 1) the initiated cells are different in the two subtypes and 2) the epithelioid subtype progresses into the sarcomatoid subtype. These hypotheses are being extensively investigated in our laboratory.

Anthophyllite in this preparation was not carcinogenic to mesothelial cells when administered intraperitoneally. The physical characteristics of the fibers used in the present study were not significantly different from those obtained from the UICC, although those from the UICC were shorter and contained a number of impurities, including talc, chlorite and phlogopite[12]. The current result of anthophyllite administration is inconsistent with the results of studies using fibers from the UICC[22] and from Matsubase, Kumamoto, Japan[23] (Table 1). This may be explained by the different asbestos fibers, the different strain of rats used, or both. Approximately 4.3% of male Fischer-344 rats show spontaneous mesothelioma of the tunica vaginalis[24]. Previous studies used female rats of Fischer-344 strain. In contrast, we used F1 rats that were hybrids of the Fischer-344 and Brown-Norway strains, in which we have not observed a single spontaneous mesothelioma[9, 25, 26]. We believe that it is the strain difference, not the impurities, that is responsible for the present results because Afghan anthophyllite is the most pure[12]. Although we cannot completely rule out carcinogenicity when the anthophyllite we used is mechanically ground to create thinner fibers, we can suggest that the carcinogenic risk of anthophyllite is lower than the three most common asbestos fibers and tremolite. The rat strain difference in asbestos sensitivity should be investigated in future studies.

In the tremolite group, we found that males were more susceptible to MM than females. This suggests the involvement of sex hormones and, presumably, the resulting difference in iron metabolism. These findings are consistent with the findings of a previous study related to chrysotile-induced mesothelial carcinogenesis, particularly in the group that received additional NTA injections[9]. Unexpectedly, NTA did not promote tremolite-induced mesothelial carcinogenesis. We observed iron deposits in the spleen and in the tissue surrounding asbestos deposits. This is most probably due to the ferroportin block via foreign body-associated inflammation[27]. Therefore, involvement of local iron overload is plausible in tremolite-induced carcinogenesis as well. We suspect that calcium within the chemical formula for tremolite ($[Ca_2Mg_5Si_8O_{22}(OH)_2]_n$) might have blocked the access of NTA[28] to iron.

There are two limitations in this study. First, the minor asbestos fibers used in the present experiments are not standardized as are the major asbestos of the UICC. Therefore, we cannot completely rule out the possibility that more finely ground anthophyllite is carcinogenic to mesothelial cells. Second, the administration of asbestos was performed intraperitoneally, providing maximum asbestos exposure to mesothelial cells. This route is not identical to that of human exposure, but it is a sensitive model.

In conclusion, short fiber preparation tremolite was potently carcinogenic to mesothelial cells. In contrast, Afghan anthophyllite in the current preparation was not carcinogenic. Thus, the fiber diameter appears to be more important than the fiber length for mesothelial carcinogenesis, although the fiber length appears more important for clearance from the lung when exposure is via the respiratory tract. Anthophyllite is also contaminated with talc deposits and chrysotile A[12]. Our results suggest these contaminants are less responsible for MM, especially for chrysotile asbestos. Genomic alteration of tremolite-induced rat MM is under investigation in our laboratory.

CONFLICTS OF INTEREST

The authors have declared that they have no conflicts of interest.

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