Table 1. Size of MWCNT-N in the preparation medium and in the lung tissue

	MWCNT-N in the preparation medium before administration	MWCNT-N in the lung tissue	
MWCNT-N Fraction		Alveolar Wall	Tumor area
Unfiltered	4.2 ± 2.9[a]	3.1 ± 1.0	2.0 ± 0.4
Flow-through	2.6 ± 1.6	2.8 ± 0.9	2.6 ± 0.6
Retained	> 2.6	3.2 ± 0.8	3.2 ± 0.7

[a]Mean ± SD μm

Table 2. Amount of MWCNT-N in the lung at weeks 2 and 109

Week 2			Week 109			
Rats[a]	MWCNT-N Fraction	Amount[b]	Rats[a]	MWCNT-N Fraction	Amount[b]	Ratio of week 109 to week 2
5	Unfiltered	605 ± 119	5	Unfiltered	486 ± 44	80%
4	Flow-through	603 ± 238	4	Flow-through	426 ± 116	71%
5	Retained	701 ± 176	5	Retained	268 ± 43	38%

[a] Number of rats examined

[b] μg MWCNT-N per gram wet-weight paraformaldehyde fixed lung tissue

1

Table 3. Incidence of pleural malignant mesothelioma and lung tumors

MWCNT Fraction	Rats	Malignant Mesothelioma	Lung Tumors			Total Tumor burden (%)
		Pericardial and/or Pleura (%)	Adenoma	Adeno-carcinoma	Combined (%)	
NT	15	0 (0.0)	0	0	0 (0.0)	0 (0.0)
V	13	0 (0.0)	0	0	0 (0.0)	0 (0.0)
NT + V	28	0 (0.0)	0	0	0 (0.0)	0 (0.0)
U	12	3[a] (25.0)	1	3[a]	4 (33.3)	7 (58.3)
FT	12	3 (25.0)	1	2	3 (25.0)	6 (50.0)
R	14	0 (0.0)	2	5	7 (50.0)	7 (50.0)
U + FT + R	38	6 (15.8)*	4	10	14 (36.8)**	20 (52.6)**

NT, No treatment; V, Vehicle; U, Unfiltered; FT, Flow-through; R, Retained

Average length: U = 4.2 ± 2.9 μm; FT = 2.6 ± 1.6 μm; R was not measurable

[a] One rat had both a malignant mesothelioma and a lung adenocarcinoma

* $p<0.05$; ** $p<0.001$ vs. the control groups (NT+V)

Table 4. Tumor incidence in other organs[a]

MWCNT Fraction	Rats	Abdominal cavity mesothelioma (%)	Leydig cell tumor (%)	Leukemia/ lymphoma (%)	Subcutaneous fibroma (%)	Pureputial gland tumor (%)	Pituitary gland tumor (%)
NT	15	0	10 (66.7)	0	4 (26.7)	0	0
V	13	2 (15.4)	10 (76.9)	2 (15.4)	2 (15.4)	1 (7.7)	1 (7.7)
NT + V	28	2 (7.1)	20 (71.4)	2 (7.1)	2 (7.1)	1 (3.6)	1 (3.6)
U	13	1 (7.7)	9 (69.2)	4 (30.8)	0	0	1 (7.7)
FT	13	1 (7.7)	8 (61.5)	3 (23.1)	1 (7.7)	1 (7.7)	2 (15.4)
R	15	0	8 (53.3)	3 (20.0)	1 (6.7)	0	1 (6.7)
U + FT + R	41	2 (4.9)	25 (61.0)	10 (24.4)	2 (4.9)	1 (2.4)	4 (9.8)

NT, No treatment; V, Vehicle; U, Unfiltered; FT, Flow-through; R, Retained

Average length: U = 4.2 ± 2.9 μm; FT = 2.6 ± 1.6 μm; R was not measurable

[a] Including 1 adrenal cortical adenoma in NT, 1 liver adenoma in FT and 1 sarcoma of unknown origin in R.

1

Table 5. Physical characteristics of MWCNT-7 and MWCNT-N

	MWCNT-7	MWCNT-N
Length	1-4 μm (38%)	1-4 μm (51%)
Length	5-20 μm (58%)	5-20 μm (47%)
Diameter	20-100 nM (98%)	30-100 nM (95%)
Layers	35 - 40	10
Iron Content	0.3 - 0.4%	0.04 - 0.05%

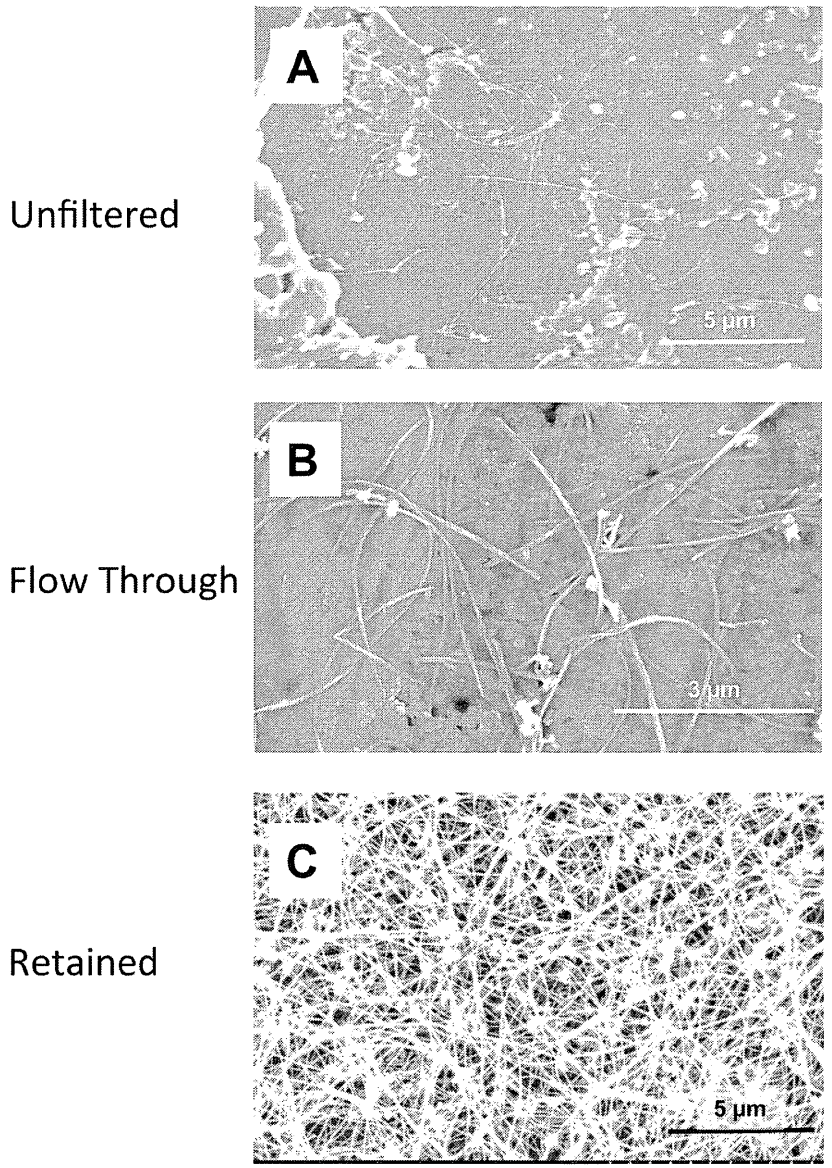

Figure 1
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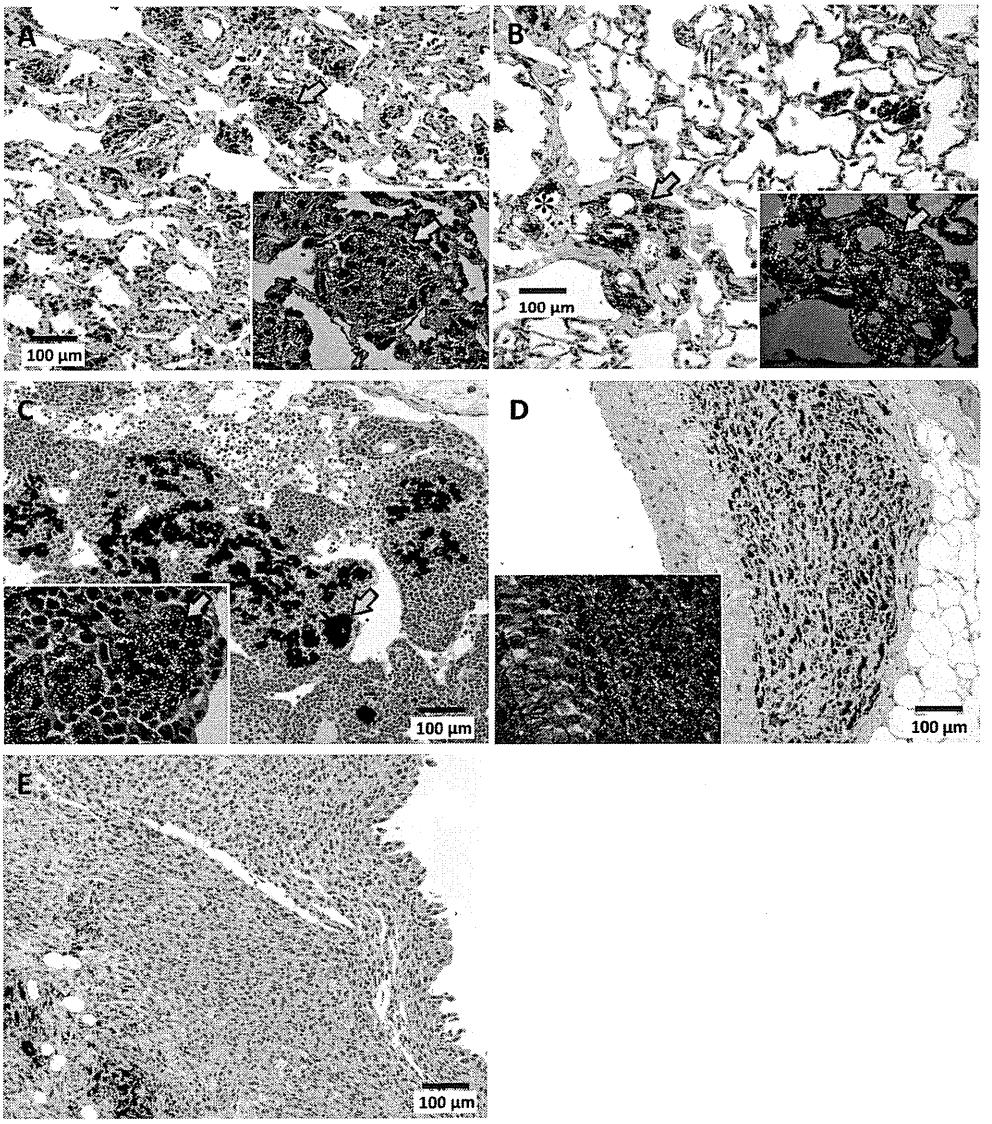

Figure 2
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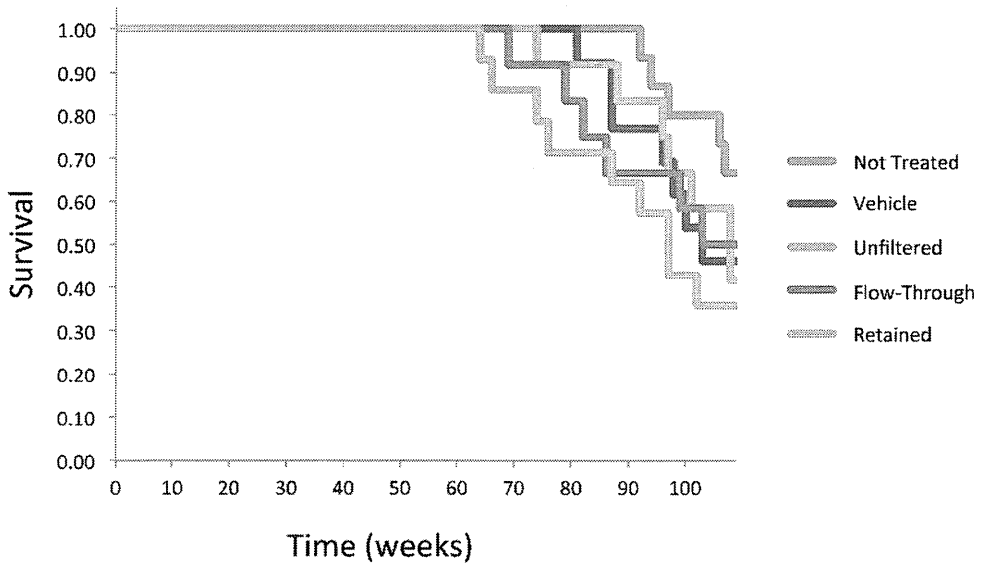

Figure 3
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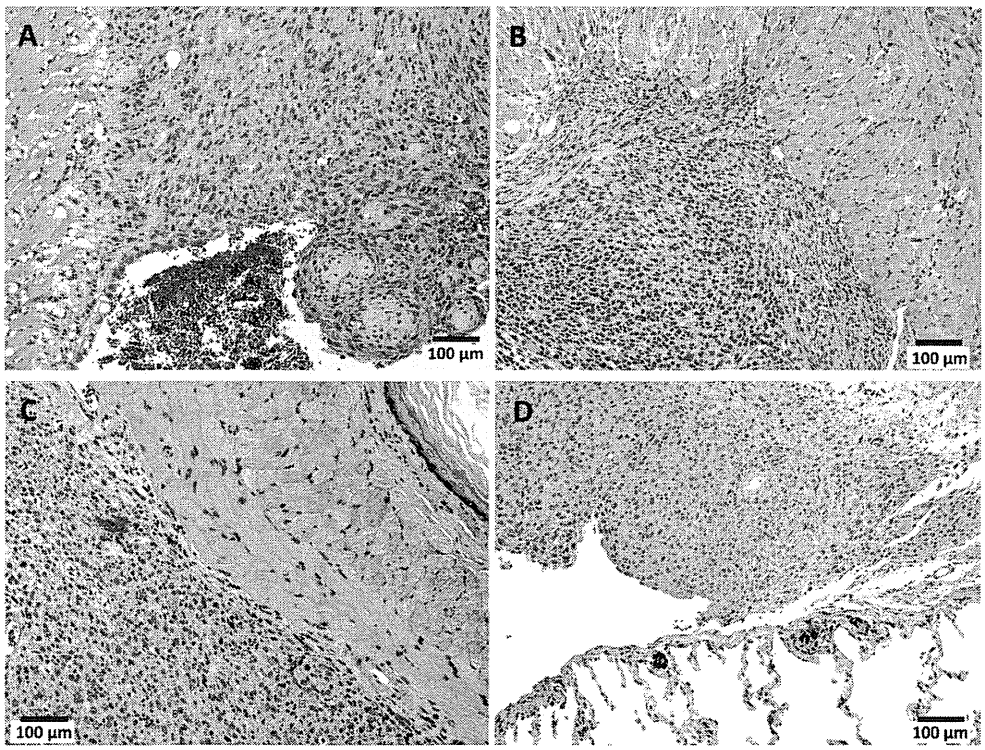

Figure 4
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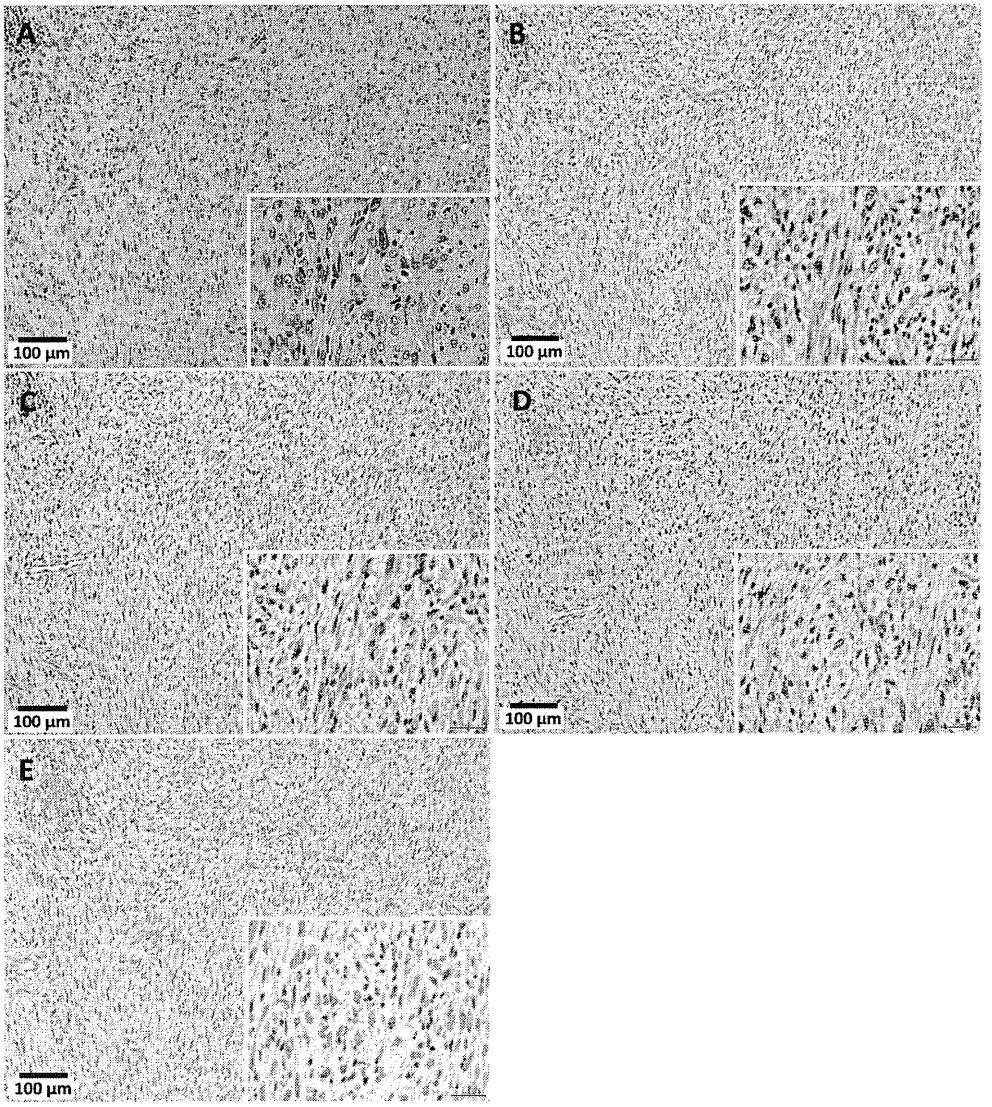

Figure 5
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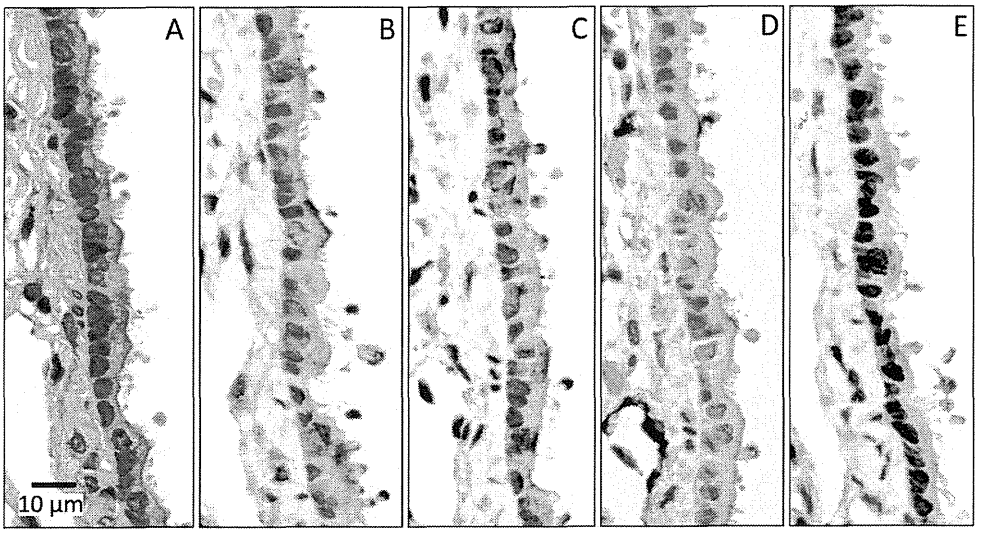

Figure 6
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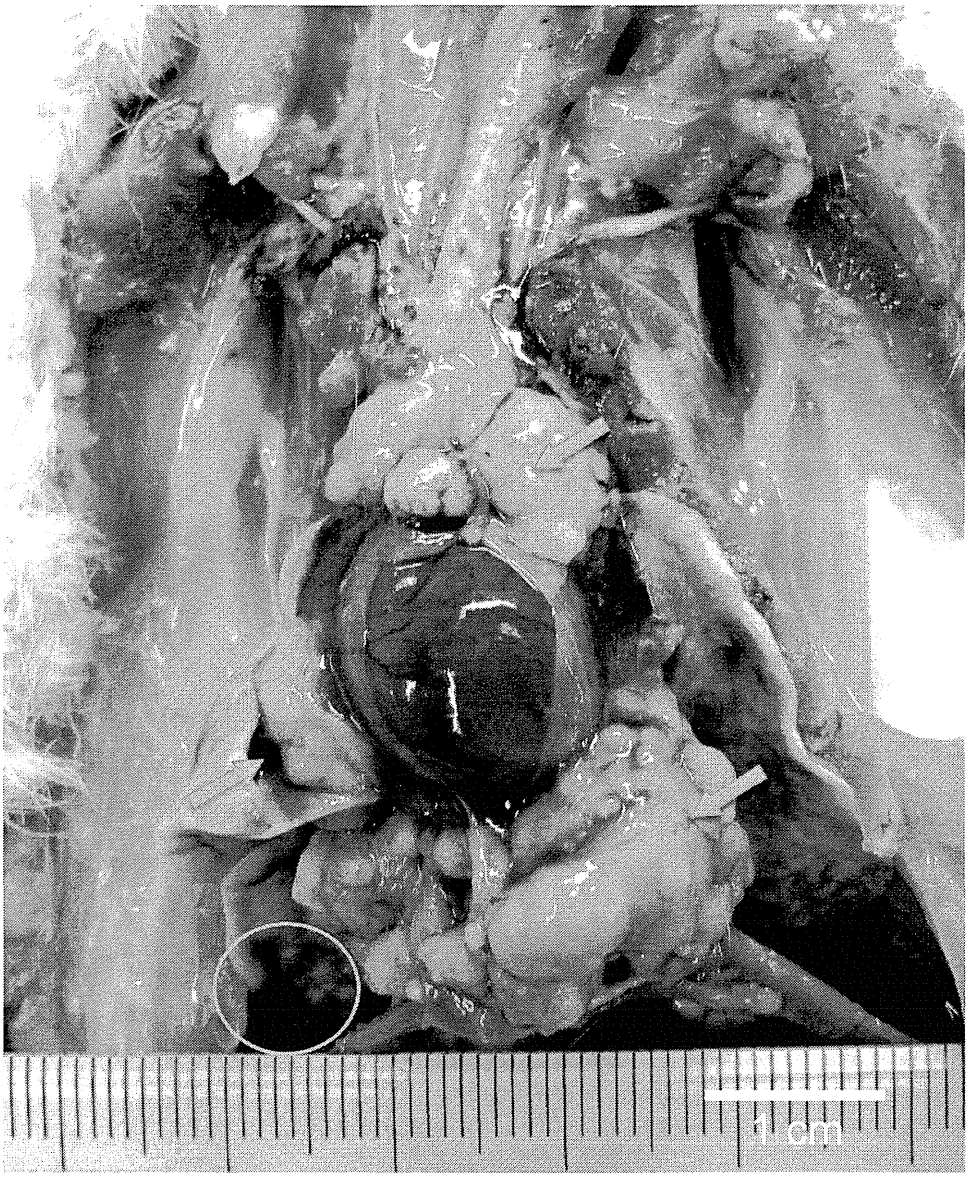

Figure 7
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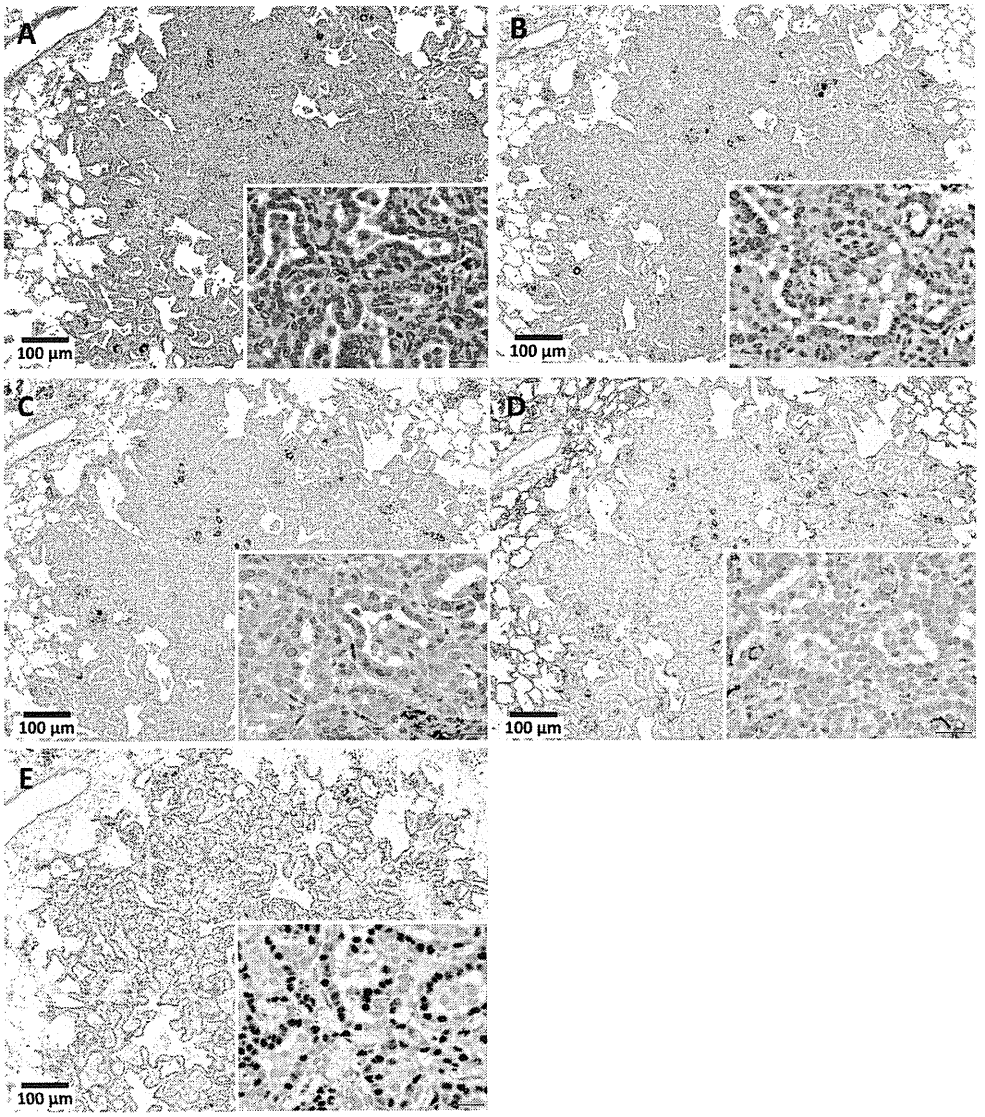

Figure 8
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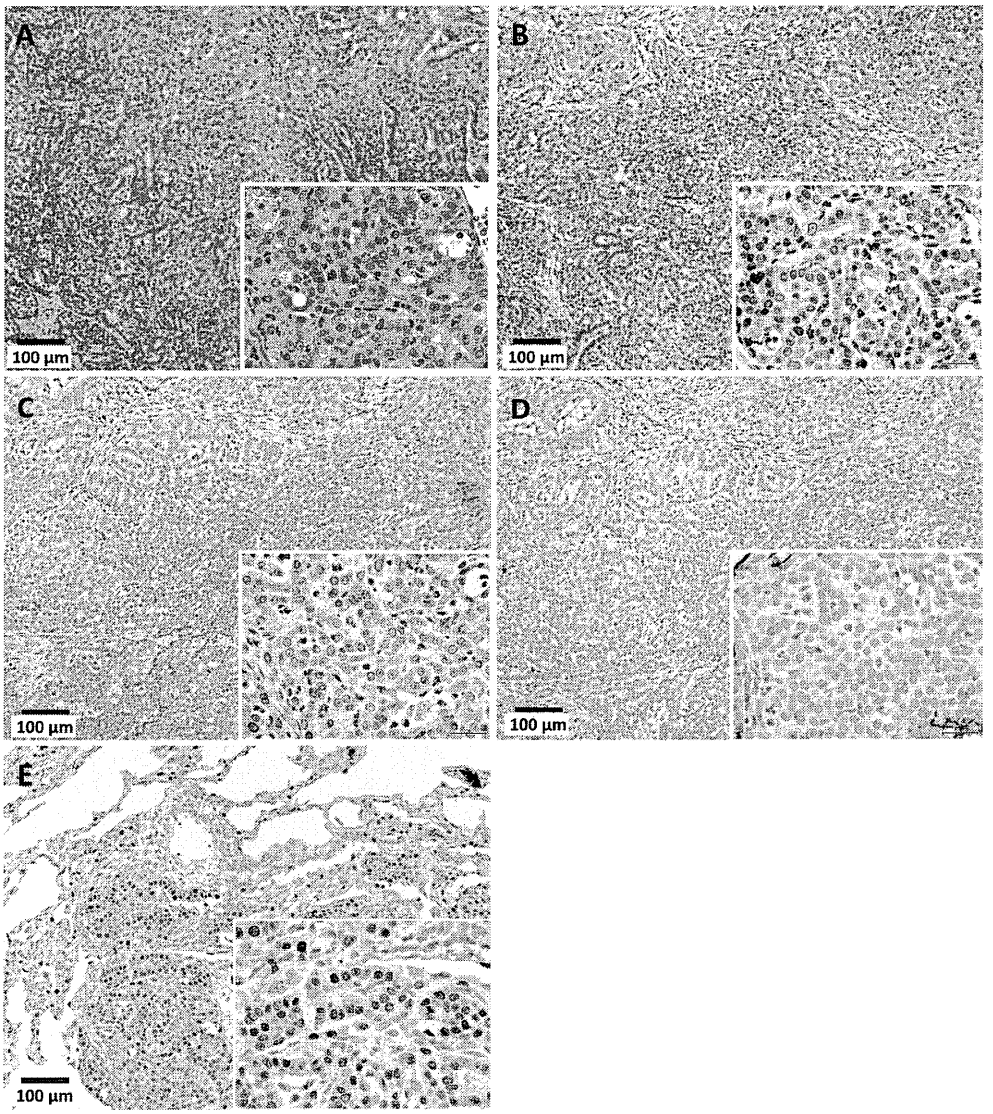

Figure 9
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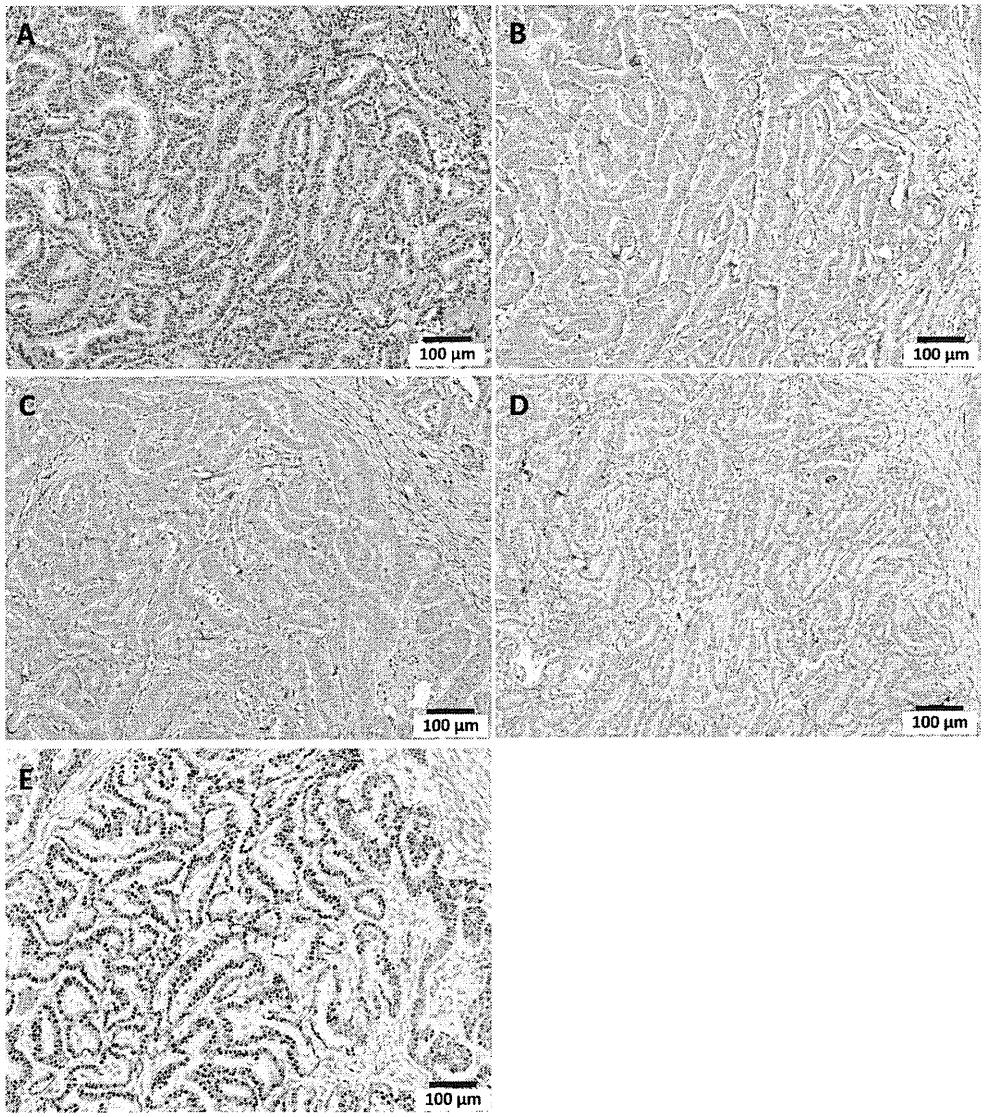

Figure 10
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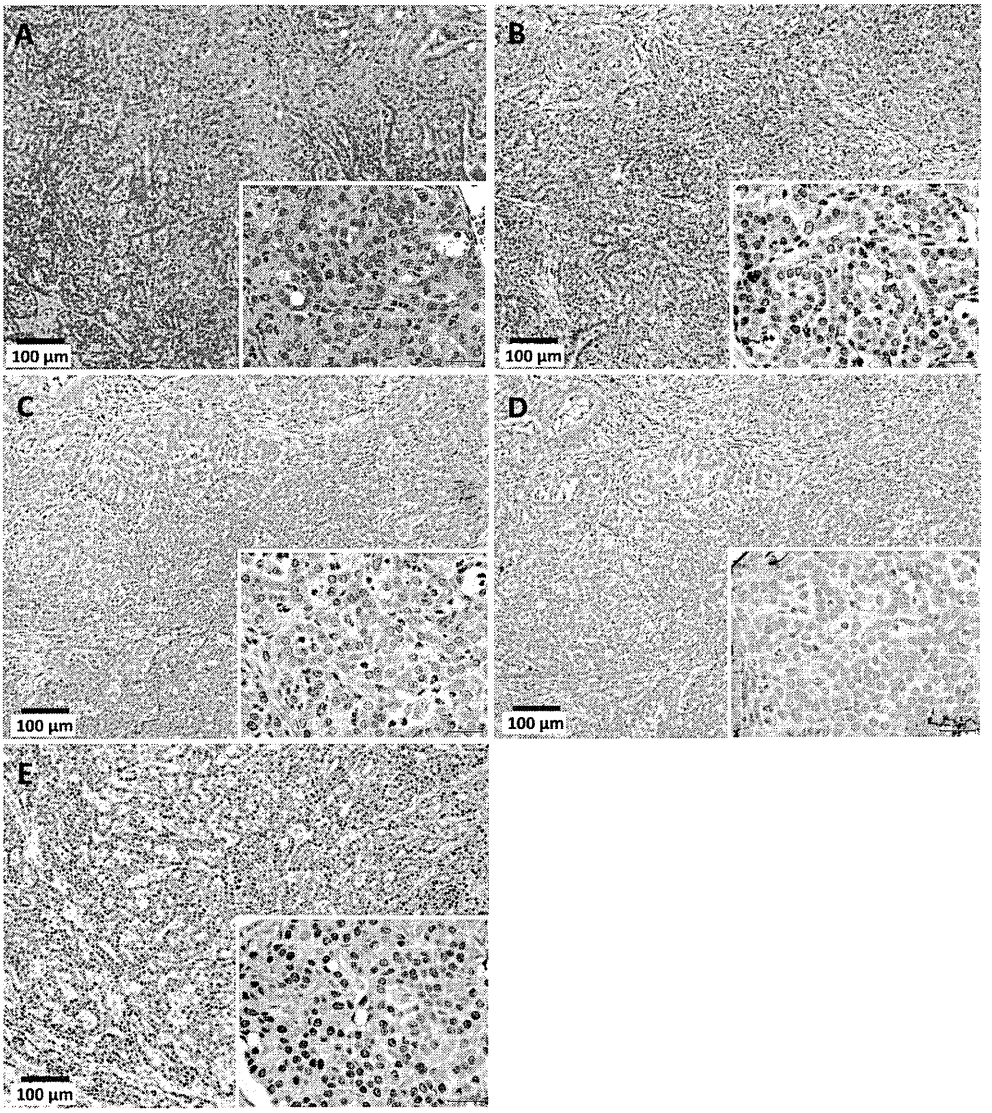

Figure 11
192x217mm (300 x 300 DPI)

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Chemokine (C-C motif) ligand 3 detection in the serum of persons exposed to asbestos: A patient-based study

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Key words

Asbestos, biological markers, chemokine CCL3, Environmental carcinogens, mesothelioma

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Exposure to asbestos results in serious risk of developing lung and mesothelial diseases. Currently, there are no biomarkers that can be used to diagnose asbestos exposure. The purpose of the present study was to determine whether the levels or detection rate of chemokine (C-C motif) ligand 3 (CCL3) in the serum are elevated in persons exposed to asbestos. The primary study group consisted of 76 healthy subjects not exposed to asbestos and 172 healthy subjects possibly exposed to asbestos. The secondary study group consisted of 535 subjects possibly exposed to asbestos and diagnosed with pleural plaque (412), benign hydrothorax (10), asbestosis (86), lung cancer (17), and malignant mesothelioma (10). All study subjects who were possibly exposed to asbestos had a certificate of asbestos exposure issued by the Japanese Ministry of Health, Labour and Welfare. For the primary study group, levels of serum CCL3 did not differ between the two groups. However, the detection rate of CCL3 in the serum of healthy subjects possibly exposed to asbestos (30.2%) was significantly higher ($P < 0.001$) than for the control group (6.6%). The pleural plaque, benign hydrothorax, asbestosis, and lung cancer groups had serum CCL3 levels and detection rates similar to that of healthy subjects possibly exposed to asbestos. The CCL3 chemokine was detected in the serum of 9 of the 10 patients diagnosed with malignant mesothelioma. Three of the patients with malignant mesothelioma had exceptionally high CCL3 levels. Malignant mesothelioma cells from four biopsy cases and an autopsy case were positive for CCL3, possibly identifying the source of the CCL3 in the three malignant mesothelioma patients with exceptionally high serum CCL3 levels. In conclusion, a significantly higher percentage of healthy persons possibly exposed to asbestos had detectable levels of serum CCL3 compared to healthy unexposed control subjects.

Inhalation of asbestos elicits a high risk of developing lung and mesothelial diseases, including fatal malignant mesothelioma. Although the production and use of asbestos is now limited in many countries, asbestos is still widely used.[(1)] In addition, due to the long latency period of asbestos-associated disease development, even in countries that have restricted the use of asbestos, past exposure remains a serious public health issue. The mortality due to malignant mesothelioma alone in the USA, Europe, Japan, and Australia, regions with strong health controls in place, is predicted to be more than 400 000 between the years 2005 and 2045,[(2)] and the yearly worldwide mortality due to all asbestos exposure-related diseases is predicted to be 100 000–140 000.[(3)] Careful follow-up of patients exposed to asbestos is a key issue in controlling the development of asbestos-associated diseases. Accordingly, identification of healthy asymptomatic persons exposed to asbestos is an

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important goal. Testing for asbestos exposure is particularly relevant for persons who work or previously worked in asbestos factories, residents who lived near asbestos factories, workers processing rubble resulting from destruction of asbestos-containing homes and buildings, and firefighters and other rescue workers.

Numerous studies searching for biomarkers of asbestos exposure and malignant mesothelioma, with the majority concentrating on malignant mesothelioma, have been carried out, and a number of markers have been proposed.[4–32] Most of these studies, however, suffer from small patient numbers, and consequently, the diagnostic value of most proposed markers requires further evaluation. Osteopontin (OPN) and soluble mesothelin-related proteins (SMRP), as defined in Cristaudo *et al.* 2011,[33] have generally been regarded as the most promising biomarkers.[13,33–42] Application of OPN, however, is limited: OPN is not able to discriminate between asbestos-exposed subjects without malignant mesothelioma and unexposed subjects,[11,34] and OPN is not specific to mesothelioma.[34,43–48] Initially, SMRP was also found to be limited to detection of malignant mesothelioma,[4,34] however, a later study reported that SMRP might also serve as a marker of asbestos exposure.[10] These conflicting results remain to be resolved. Another promising biomarker is fibulin-3;[22] however, fibulin-3 cannot distinguish asbestos-exposed subjects without malignant mesothelioma from unexposed subjects.[22] Therefore, establishment of biomarkers that detect asbestos exposure, and consequently identify persons at risk of developing asbestos-associated diseases, including malignant mesothelioma, remains an important goal.

In rats treated with nanoscale titanium dioxide by intrapulmonary instillation, macrophages interact with TiO_2 aggregates in the lung and produce chemokine (C-C motif) ligand 3 (CCL3), also known as macrophage inflammatory protein 1-α, resulting in increased levels of CCL3 in the blood.[49] Based on this finding, we undertook the current patient-based study to determine whether the serum levels or the detection rate of CCL3 are elevated in asbestos-exposed subjects.

In this study, we determined the serum CCL3 levels in healthy asymptomatic subjects possibly exposed to asbestos and in healthy unexposed subjects. We also determined the serum CCL3 levels in patients possibly exposed to asbestos and diagnosed with pleural plaque, benign hydrothorax, asbestosis, lung cancer, and malignant mesothelioma. Our primary finding was that a significantly higher percentage of healthy asymptomatic persons possibly exposed to asbestos had detectable levels of serum CCL3 compared to healthy unexposed control subjects.

Materials and Methods

Ethics statement. This study was approved by the Ethics Review Committee of the respective participating institutes and hospitals: Nagoya City University Graduate School of Medical Sciences (Nagoya, Japan), Asahi Rosai Hospital (Owariasahi, Japan), Saiseikai Chuwa Hospital (Sakura, Japan), and Nagoyashi (Nagoya City) Koseiin Medical Welfare Center (Nagoya, Japan), and conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Tokyo 2004). Participants provided written informed consent before inclusion in the study, after which serum samples were obtained, coded, and stored in aliquots at −80°C until use.

Subjects. *Serum of unexposed subjects.* Control sera were collected from the teaching and research staff at the Nagoya City University Medical School and healthy inmate residents /patients at Nagoyashi Koseiin Medical Welfare Center Hospital (Koseiin Hospital) (n = 76; mean age, 50.9 ± 17.7 years). These subjects had no history of work or tenancy at asbestos-related workplaces or residences. They were free from lung and pleural lesions on periodical (once or twice a year) institutional health examinations including physical, chest x-ray, blood biochemical, and electrocardiogram examinations.

Serum of exposed subjects. The sera of subjects possibly exposed to asbestos (n = 707; mean age, 69.1 ± 8.2 years) were collected from patients who visited or were hospitalized in the Japan Labour Health and Welfare Organization Asahi Rosai (work-related accident) Hospital, the Saiseikai Chuwa Hospital, or the Nagoya City University Hospital from 2008 to 2012. All of the enrolled subjects potentially exposed to asbestos had certified documents issued by the Japanese Ministry of Health, Labour and Welfare for the compensation of medical care. The exposed subjects were grouped as follows: no detectable lesions (n = 172), pleural plaque (including 12 cases of pneumoconiosis complication) (n = 412), benign hydrothorax (n = 10), asbestosis lung (asbestosis) (n = 86), lung cancer (n = 17), and malignant mesothelioma (n = 10). The diagnosis for all lung and mesothelial disease cases was made by chest x-ray and/or computed tomography examinations. The diagnosis of malignant tumors was made by endoscopic examination coupled with histopathological examination of biopsy specimens. Pathological examination of malignant mesothelioma included an immunohistochemical antibody panel; positive markers were calretinin, mesothelin, WT1 (Wilms tumor 1), D2-40 (mAb directed against M2A antigen), and CK5/6 (cytokeratin 5/6). For malignant mesothelioma diagnosis, staining with at least two positive markers must be positive and carcinoembryonic antigen must be negative. In addition, thyroid transcription factor 1 and Ber-EP4 staining should be negative (see also ref. 50). All the malignant mesothelioma cases were epithelial-type tumors. For all subjects, job history and the site of residence were recorded. Residents near asbestos factories without any history of asbestos-related occupation were certified as asbestos-exposed and included in the asbestos-exposed groups. For smokers, previous or current smoking status was recorded and expressed as smoking index (Brinkman index: daily number of cigarettes × years of smoking).

Enzyme-linked immunosorbent assay. Human serum CCL3 was measured using the Quantikine Human CCL3/MIP-1a Immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, except that the samples added to the ELISA plates were incubated at room temperature for 3 h instead of 2 h. Levels of CCL3 below the detection limit (7.8 pg/mL) were arbitrarily regarded as 0. The association of CCL3 levels with the subject's work place, work duties, length of exposure, lapse of time after the last exposure, and smoking habit was analyzed.

Immunohistochemistry of malignant mesothelioma. Four biopsy cases and one autopsy case with malignant mesothelioma were available and examined by immunohistochemistry for the presence of CCL3, C-ERC/mesothelin (mesothelin), and CD68, a macrophage marker. (The autopsy case and three of the four biopsy cases were available for analysis of serum CCL3.) Slides of malignant mesothelioma were deparaffinized and heated in 10 mM sodium citrate, 0.05% Tween 20 (pH 6.0) for 10 min for antigen retrieval. The slides were blocked with Blocking One (03953-95; Nacalai Tesque, Kyoto, Japan) and incubated with rabbit anti-human CCL3 polyclonal antibodies (LS-B1056; Lifespan Biosciences, Seattle, WA, USA)

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diluted 1:100 at 4°C overnight and then washed and incubated with Alexa Fluor 488 labeled anti-rabbit secondary antibodies diluted 1:500 (Invitrogen Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. The slides were then washed with Blocking One for 30 min and incubated with rabbit anti-human mesothelin mAbs (ab93620; Abcom, Tokyo, Japan) diluted 1:100 at 4°C overnight, and then washed and incubated with Alexa Fluor 546 labeled anti-rabbit secondary antibodies (Invitrogen Molecular Probes) diluted 1:500 for 1 h at room temperature.

Statistics. The Kruskal–Wallis test was used to analyze the levels of CCL3 in the serum. Spearman's rank correlation coefficient was used to analyze the associations of CCL3 level and background factors: age, gender, cigarette consumption (scored by the Brinkman index), the length of exposure time to asbestos, and the lapse of time after the last potential exposure to asbestos. The Steel–Dwass method was used to compare CCL3 levels among the asbestos-exposed subgroups: no lesion, pleural plaque, benign hydrothorax, asbestosis lung, lung cancer, and malignant mesothelioma. The effects of background factors on detection of serum CCL3 was analyzed using multivariable logistic regression, and CCL3 detection was analyzed using multivariable logistic regression adjusted by background factors. P-values < 0.05 were considered to indicate statistical significance. Statistical analyses were carried out using JMP version 9.0 (SAS Institute, Cary, NC, USA).

Results

Study population. The primary study population was composed of 76 healthy subjects not exposed to asbestos and 172 healthy, asymptomatic (i.e., no detectable lung or pleural lesions) patients possibly exposed to asbestos. The general characteristics of the primary study group are summarized in Table 1a.

The secondary study population was composed of 535 subjects possibly exposed to asbestos and diagnosed with pleural plaque (412), asbestosis (86), benign hydrothorax (10), lung cancer (17), and malignant mesothelioma (10). The general characteristics of the secondary study group are summarized in Table 1b.

Table 1. General characteristics of the (a) primary study group, consisting of healthy subjects exposed or not exposed to asbestos (b) secondary study group, composed of subjects possibly exposed to asbestos and diagnosed with lung disease

	Diagnosis	No.	Gender		Age, years
			Male	Female	
(a)					
Unexposed	No lesions	76	48	28	50.9 ± 17.7
Asbestos exposed	No lesions	172	141	31	65.7 ± 8.8
(b)					
Exposed to asbestos	Pleural plaque†	412	315	97	69.7 ± 7.8
	Asbestosis	86	67	19	71.6 ± 6.9
	Benign hydrothorax	10	9	1	70.5 ± 6.2
	Lung cancer	17	17	0	73.5 ± 7.4
	Malignant mesothelioma	10	9	1	69.9 ± 5.6

†Includes 12 cases of pleural plaque with pneumoconiosis (mainly silicosis).

All study participants, with the exception of the 76 healthy subjects not exposed to asbestos, had certificates of asbestos exposure issued by the Japanese Ministry of Health, Labour and Welfare. However, confirmation of the presence of asbestos fibers in the lung or pleural tissues of healthy, asymptomatic persons is not possible. Therefore, in the primary study group the study participants with certificates of asbestos exposure must be assumed to be possibly exposed to asbestos, resulting in this study group being composed of an above average number of persons exposed to asbestos rather than being composed entirely of asbestos-exposed persons. Consequently, these study subjects are referred to as healthy, asymptomatic subjects possibly exposed to asbestos in this report.

Serum CCL3 levels: Primary study group. The serum CCL3 levels in the unexposed group and the healthy, asymptomatic subjects possibly exposed to asbestos are shown in Figure 1. For the study participants with detectable serum CCL3, there was no difference in CCL3 levels between the healthy control subjects and the healthy, asymptomatic subjects possibly exposed to asbestos. The study data can be downloaded from Table S1.

Serum CCL3 levels and background factors: Primary study group. Age and cigarette consumption (scored by the Brinkman index) showed a significant association with serum CCL3 levels (Table 2). Gender, the length of exposure time to asbestos, and the lapse of time after the last potential exposure to asbestos did not show a significant association with serum CCL3 levels.

Detection of serum CCL3: Primary study group. Subjects with CCL3 levels higher than 7.8 pg/mL, the detection limit of the ELISA assay, were defined as positive for serum CCL3. The detection rate of CCL3 in the serum of the primary study group is shown in Table 3. The detection rate of serum CCL3 in healthy, asymptomatic subjects possibly exposed to asbestos (52/172; 30.2%) was significantly higher (see Table 4) than in the unexposed control group (5/76; 6.6%).

Detection of serum CCL3 and background factors: Primary study group. Age, gender, smoking habit (never, previous, or current smoker), cigarette consumption (scored by the Brinkman index), the length of exposure time to asbestos, and the lapse of time after the last exposure to asbestos did not show a significant association with detection of CCL3 in the serum.

Levels of CCL3: Secondary study group. The serum CCL3 levels in the secondary study population are shown, alongside the levels in the primary study population, in Figure 2. For the study participants with detectable serum CCL3 in the pleural plaque, asbestosis, benign hydrothorax, and lung cancer groups, CCL3 levels were not different between groups or from the healthy, asymptomatic subjects possibly exposed to asbestos. In contrast, detectable serum CCL3 levels in the 10 patients constituting the mesothelioma group were significantly higher compared to the other groups. Notably, the higher levels of serum CCL3 in the mesothelioma group was entirely due to the levels in three patients with extraordinarily high – 611, 1007, and 2012 pg/mL – serum CCL3 levels. The study data can be downloaded from Table S1.

Detection of serum CCL3: Secondary study group. The detection rate of CCL3 in the serum of the study subjects with pleural plaque (139/412; 33.7%), asbestosis (34/86; 39.5%), benign hydrothorax (3/10; 30.0%), and lung cancer (5/17; 29.4%) was similar to that of the healthy, asymptomatic subjects possibly exposed to asbestos (Table 5): there were no

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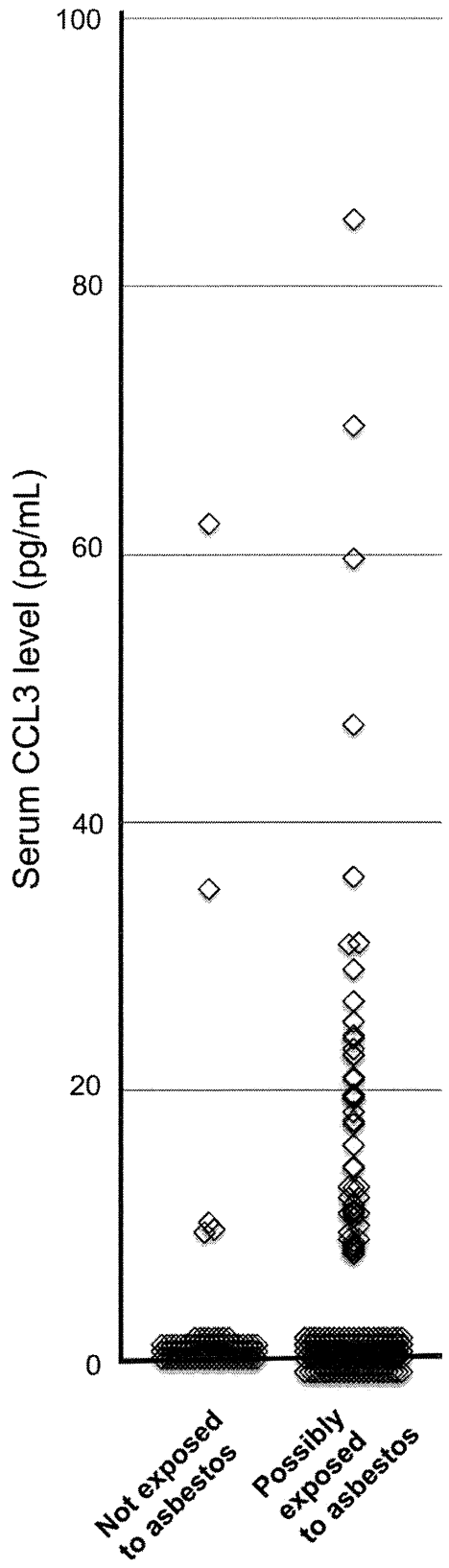

Fig. 1. Serum chemokine (C-C motif) ligand 3 (CCL3) levels in the primary study group. Levels of CCL3 in subjects not exposed to asbestos (controls) and in healthy, asymptomatic subjects possibly exposed to asbestos. Excluding the subjects without detectable CCL3, serum CCL3 levels in the group composed of healthy, asymptomatic subjects possibly exposed to asbestos are not different from the five control participants with detectable CCL3 in their serum.

Table 2. Associations between chemokine (C-C motif) ligand 3 levels with background factors

Background factor	Rho	*P*-value
Age	+0.196	0.002
Cigarette consumption	+0.171	0.026
Gender (M = 0; F = 1)	−0.052	0.417
Length of exposure time	+0.070	0.431
Lapse of time since last exposure	−0.080	0.359

F, female; M, male.

Table 3. Detection of serum chemokine (C-C motif) ligand 3 in the primary study group, consisting of healthy subjects exposed or not exposed to asbestos

Lesion category	Total number of subjects	Number of positive subjects	Detection rate, %	95% confidence interval
Unexposed				
No lesions	76	5	6.6	0.3–14.5
Asbestos exposed				
No lesions	172	52	30.2	23.9–37.5

Table 4. Odds ratio for asbestos exposure. The detection rate of serum CCL3 in healthy, asymptomatic subjects possibly exposed to asbestos was significantly higher than in the unexposed control group

	Odds ratio	95% confidence interval	*P*-value
No lesion group/control	6.15	2.56–18.3	<0.001

significant differences in the detection rate of serum CCL3 between any of these groups. In contrast, the detection rate of CCL3 in the serum of the 10 patients constituting the mesothelioma group (9/10) was significantly higher than in the other groups (Table 5).

Immunohistochemical localization of CCL3 in malignant mesotheliomas. All biopsy specimens (4) and the autopsy specimen (1) showed clear expression of CCL3 in the tumor cells. In Figure 3, panel A is a malignant mesothelioma with glandular formation, and panel B is a malignant mesothelioma with solid proliferation. The tumor cells co-express CCL3 and mesothelin with CCL3 localizing primarily to the cytoplasm and mesothelin localizing more to the plasma membrane. These specimens were negative for the macrophage marker CD68 (data not shown). The levels of serum CCL3 of these two cases were 40.2 (panel A) and 2012.4 (panel B) pg/mL.

Discussion

Asbestos has a long history of use worldwide, and annual global production of asbestos remains at over 2 million tons.[3] The extensive use of asbestos has resulted in widespread risk of developing asbestos-associated diseases due to deposition of asbestos in the lung and pleural tissue, which can persist for the remainder of the exposed person's lifetime, causing foreign body inflammation in the lung and pleura. The ability to iden-

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