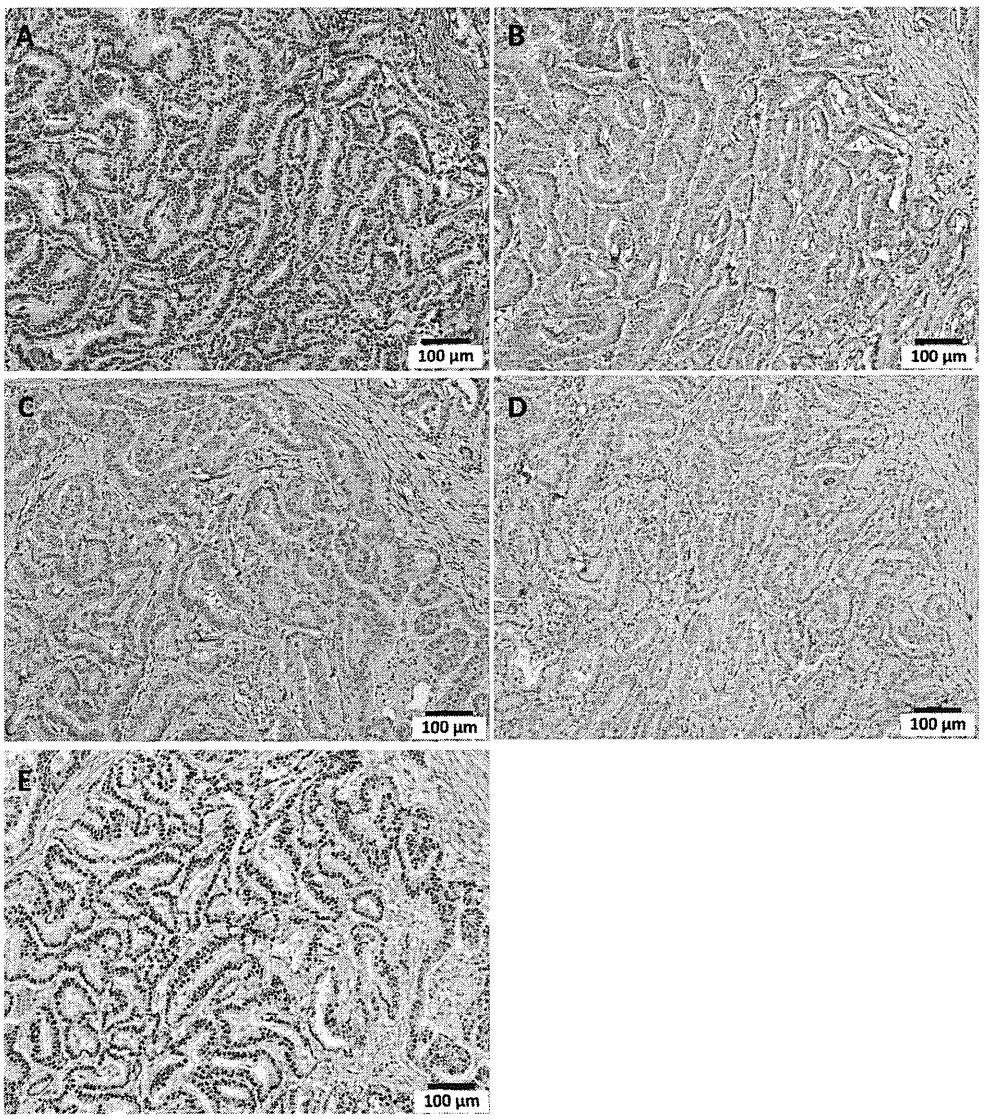

Figure 10
216x245mm (300 x 300 DPI)

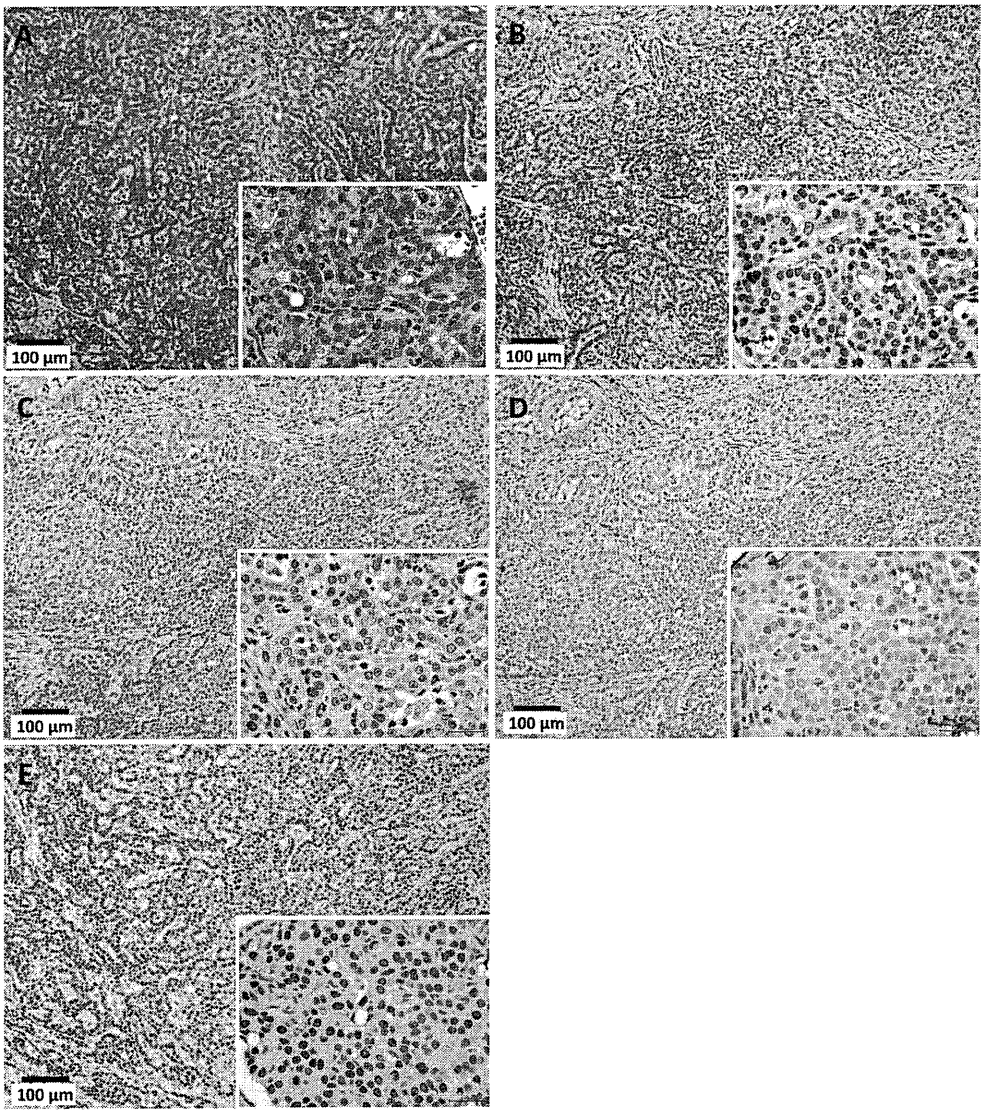

Figure 11
192x217mm (300 x 300 DPI)

Cancer Science
Japanese Cancer Association JCA
Open Access

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

Jiegou Xu,[1,2] David B. Alexander,[1] Masaaki Iigo,[1] Hirokazu Hamano,[3] Satoru Takahashi,[4] Takako Yokoyama,[5] Munehiro Kato,[5] Ikuji Usami,[5] Takeshi Tokuyama,[6] Masahiro Tsutsumi,[7] Mouka Tamura,[8] Tetsuya Oguri,[9] Akio Niimi,[9] Yoshimitsu Hayashi,[10] Yoshifumi Yokoyama,[10] Ken Tonegawa,[11] Katsumi Fukamachi,[12] Mitsuru Futakuchi,[12] Yuto Sakai,[12] Masumi Suzui,[12] Michihiro Kamijima,[13] Naomi Hisanaga,[14] Toyonori Omori,[15] Dai Nakae,[16] Akihiko Hirose,[17] Jun Kanno[18] and Hiroyuki Tsuda[1]

[1]Nanotoxicology Project, Nagoya City University, Nagoya, Japan; [2]Department of Immunology, College of Basic Medical Sciences, Anhui Medical University, Hefei, China; [3]Nutritional Science Institute, Morinaga Milk Industry Co., Ltd., Zama; [4]Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya; [5]Department of Respiratory Medicine, Asahi Rosai Hospital, Owariasahi; Departments of [6]Internal Medicine; [7]Pathology, Saiseikai Chuwa Hospital, Sakura; [8]Department of Internal Medicine, Nara Medical Center, National Hospital Organization, Nara; [9]Diivision of Respiratory Medicine, Allergy and Rheumatology, Nagoya City University Hospital, Nagoya; Departments of [10]Medicine; [11]Physical Medicine and Rehabilitation, Nagoya-Shi Koseiin Medical Welfare Center, Nagoya; Departments of [12]Molecular Toxicology; [13]Occupational and Environmental Health, Nagoya City University Graduate School of Medical Sciences, Nagoya; [14]Center for Campus Health and Environment, Aichi University of Education, Kariya; [15]Department of Health Care Policy and Management, Nagoya City University Graduate School of Medical Sciences, Nagoya; [16]Department of Nutritional Science and Food Safety, Faculty of Applied Biosciences, Tokyo University of Agriculture, Tokyo; Divisions of [17]Risk Assessment, Biological Safety Research Center; [18]Cellular and Molecular Toxicology, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan

Key words

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

Correspondence

Hiroyuki Tsuda, Nanotoxicology Project, Nagoya City University, 3-1 Tanabedohri, Mizuho-ku, Nagoya 467-8603, Japan.
Tel: +81-52-836-3496; Fax: +81-52-836-3497;
E-mail: htsuda@phar.nagoya-cu.ac.jp

Funding Information

Ministry of Health, Labour and Welfare, Japan. Princess Takamatsu Cancer research Fund.

Received December 15, 2014; Revised April 14, 2015; Accepted April 27, 2015

Cancer Sci **106** (2015) 825–832

doi: 10.1111/cas.12687

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

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)

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

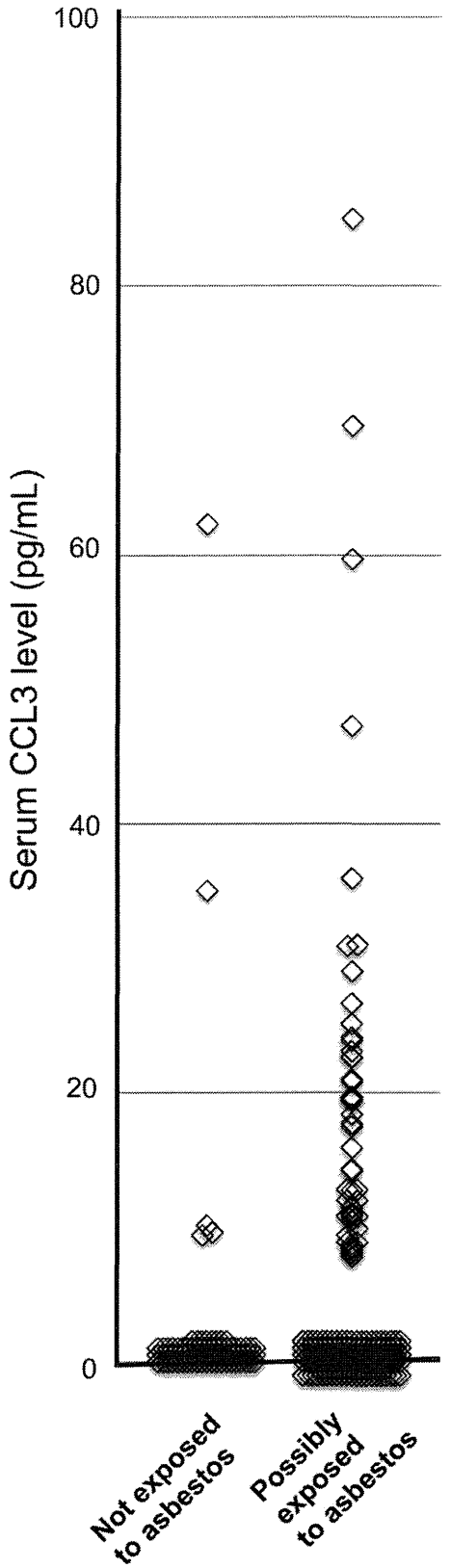

Fig. 1. Serum chemokine (C-C motif) ligand 3 (CCL3) levels in the primary study group. Levels of CCL3 in subjects not exposed to asbestos (controls) and in healthy, asymptomatic subjects possibly exposed to asbestos. Excluding the subjects without detectable CCL3, serum CCL3 levels in the group composed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

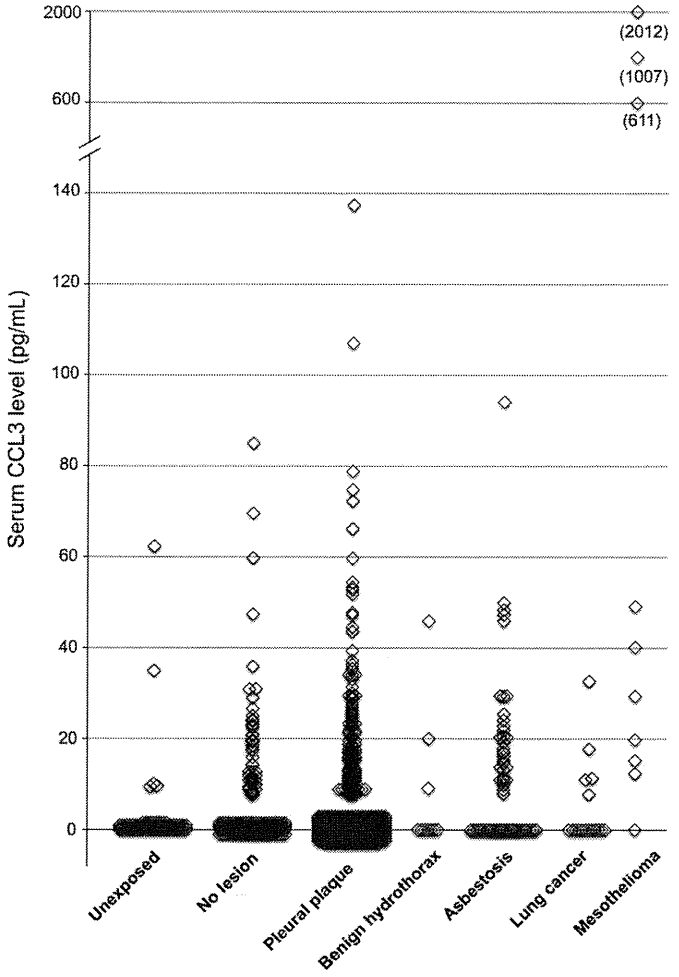

Fig. 2. Serum chemokine (C-C motif) ligand 3 (CCL3) levels in the secondary study group. Levels of CCL3 in study participants possibly exposed to asbestos and diagnosed with pleural plaque, benign hydrothorax, asbestosis, lung cancer, and mesothelioma are shown. For ease of comparison, the primary study group is shown alongside the secondary study group. The upper region of the graph is a log plot and is not continuous with the lower region of the graph. The serum CCL3 levels of the three patients plotted in the upper region are shown. Excluding the subjects without detectable CCL3 and the three subjects with exceptionally high levels of CCL3, there are no differences in CCL3 levels between any of the groups.

Table 5. Detection of serum chemokine (C-C motif) ligand 3 in the secondary study group, composed of subjects possibly exposed to asbestos and diagnosed with lung disease

Lesion category	Total number of subjects	Number of positive subjects	Detection rate, %	95% confidence interval
Exposed to asbestos				
Pleural plaque†	412	143	34.7	30.3–39.4
Asbestosis	86	34	39.5	29.9–50.1
Benign hydrothorax	10	3	30.0	10.8–60.3
Lung cancer	17	5	29.4	13.3–53.1
Malignant mesothelioma	10	9	90.0	59.6–98.2

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

tify asymptomatic persons exposed to asbestos is obviously advantageous.

The purpose of the present study was to determine whether the levels or detection rate of CCL3 in the serum were elevated in asbestos-exposed individuals. In study subjects with detectable levels of serum CCL3, the levels were not elevated in asbestos-exposed subjects compared to unexposed subjects. In contrast to serum levels, the detection rate of CCL3 in the serum of healthy, asymptomatic subjects possibly exposed to asbestos was significantly higher than in unexposed healthy subjects: the detection rate in healthy, asymptomatic subjects possibly exposed to asbestos was 30.2% and the detection rate in healthy unexposed subjects was 6.6% (see Table 3). Serum CCL3 detection rate was independent of 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.

Although the detection rate of CCL3 in the serum of healthy, asymptomatic subjects possibly exposed to asbestos was significantly higher than in the serum of unexposed control subjects, a detection rate of 30% suggests that use of serum CCL3 as a biomarker for asbestos exposure is limited. Importantly, however, verification of the presence of asbestos fibers in the lung of a healthy asymptomatic person requires microscopic examination of biopsy or autopsy specimens; therefore, confirmation of the presence of asbestos fibers in the lung or pleural tissues of a healthy, asymptomatic person possibly exposed to asbestos is not carried out. Accordingly, healthy, asymptomatic study participants with certificates of asbestos exposure must be assumed to be possibly exposed to asbestos, resulting in the asbestos-exposed no lesion study group being composed of an above average number of individuals exposed to asbestos rather than being composed entirely of asbestos-exposed individuals, and it is very unlikely that all, or even most, of the subjects in the asbestos-exposed no lesion group actually have asbestos fibers in their lungs or pleural tissues. Consequently, the true percentage of these study participants with internalized asbestos fibers having detectable levels of serum CCL3 is unknown, but it is likely to be higher, probably much higher, than 30%. Therefore, detection of CCL3 in the serum could be a valuable adjunct marker for diagnosis of asbestos exposure.

The CCL3 chemokine is produced by macrophages and other immune cells in response to infection and various other insults; therefore, obtaining multiple blood samples from a patient is needed for proper evaluation of serum CCL3 levels. It is likely that if multiple samples are obtained, the detection of serum CCL3 in healthy unexposed subjects would decrease. As detection of serum CCL3 cannot distinguish between asbestos exposure and other disease states that result in increased levels of serum CCL3, use of CCL3 as an adjunct marker of asbestos exposure is appropriate only for asymptomatic individuals.

Frustrated phagocytosis of asbestos fibers is likely to play a role in asbestos-associated diseases.[51] In addition, our previous study in rats showed that intrapulmonary dosed nanoscale titanium dioxide particles were phagocytosed by alveolar macrophages and these macrophages secreted CCL3, resulting in elevated levels of CCL3 in the serum of rats treated with titanium dioxide.[49] These data suggest that the elevation of serum CCL3 in asbestos-exposed subjects is probably due to a persistent inflammatory reaction of macrophages interacting

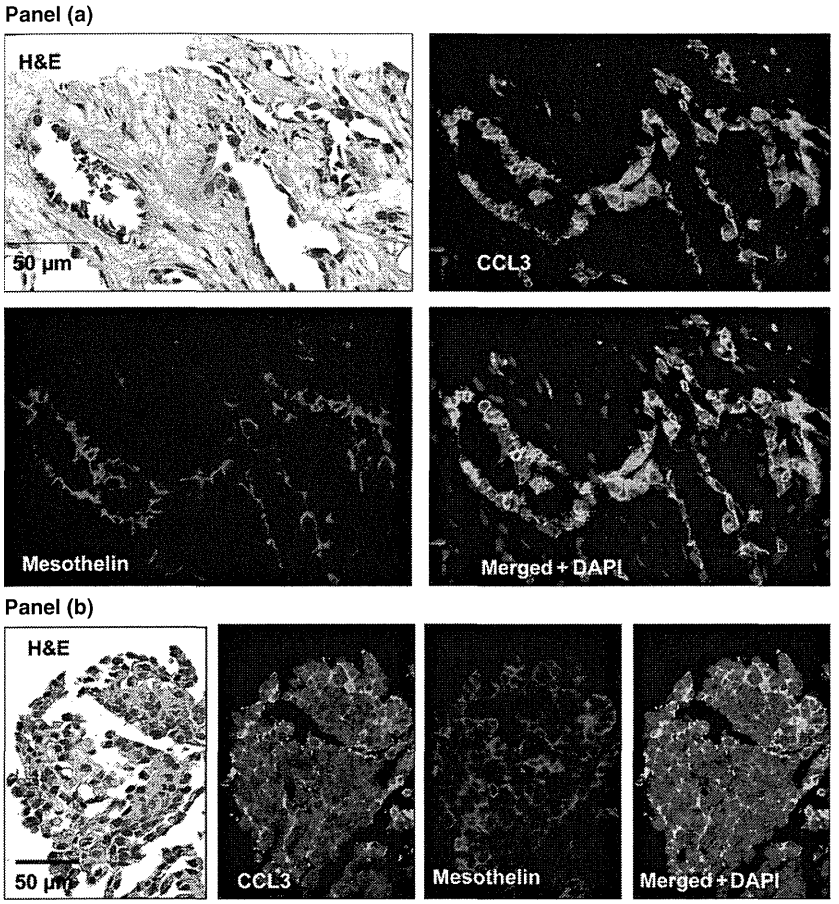

Fig. 3. Chemokine (C-C motif) ligand 3 (CCL3) and mesothelin in two biopsy specimens from patients with malignant mesothelioma. (a) Immunofluorescent staining of CCL3 and mesothelin in an enrolled biopsy case of malignant mesothelioma with glandular formation (serum CCL3, 40.2 pg/mL). Although CCL3 and mesothelin are coexpressed in the majority of tumor cells, the intracellular localization of CCL3 and mesothelin are distinct: mesothelin is localized primarily in the periphery of the tumor cells, whereas CCL3 is primarily cytoplasmic. (b) Immunofluorescent staining of CCL3 and mesothelin in an enrolled biopsy case of malignant mesothelioma with solid proliferation (serum CCL3, 2012.4 pg/mL). Similar to glandular-type malignant mesothelioma (a), most of the tumor cells expressed both CCL3 and mesothelin. Again, mesothelin is localized primarily at the cell periphery and CCL3 is more cytoplasmic.

with asbestos fibers even after the cessation of exposure to airborne asbestos.

Notably, the detection rate of CCL3 in the serum of the asbestos-exposed pleural plaque and asbestosis groups was not significantly different from the asbestos-exposed no lesion group. As the percentage of subjects diagnosed with pleural plaque and asbestosis that have asbestos fibers in their lung or pleural tissues is likely to be much higher than for the healthy, asymptomatic subjects possibly exposed to asbestos that constitute the asbestos-exposed no lesion group, this suggests that the detection rate of CCL3 in the serum of asbestos-exposed persons with certain asbestos-associated diseases is lower than for asymptomatic asbestos-exposed persons. One possible explanation for this seeming anomaly is that development of fibrotic tissue associated with inhaled asbestos fibers can prevent interaction of macrophages with the fibers.

Our study included 10 patients diagnosed with malignant mesothelioma. While this small number of malignant mesothelioma patients precludes assessment of CCL3 as a marker of malignant mesothelioma, two important observations were made. First, biopsy and autopsy specimens of malignant mesothelioma showed CCL3 expression by the tumor cells (see Fig. 3). Second, 9 of the 10 patients with malignant mesothelioma had detectable serum CCL3, and serum CCL3 levels in three of the malignant mesothelioma patients were dramatically higher than in the other study subjects. These observations suggest that expression of CCL3 by malignant mesothelioma tumor cells may be a source of the CCL3 in the serum of these patients and may result in extremely high levels of CCL3 in the serum of some malignant mesothelioma patients. We are currently accessing more malignant mesothelioma cases and appropriate controls to investigate the possibility that CCL3 may be a biomarker for malignant mesothelioma. The use of CCL3 in the pleural fluid and biopsy specimens for diagnosis of mesothelioma is also being examined.

In conclusion, inhalation of asbestos elicits a high risk of developing lung and mesothelial diseases, including fatal malignant mesothelioma. 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 important goal. The CCL3 chemokine is detectable in the serum of a significant percentage of asymptomatic persons exposed to asbestos. Persistent detection over time of CCL3 in the serum of a healthy individual possibly exposed to asbestos

can be used as an adjunct marker of asbestos exposure, and consequently as a risk marker for developing asbestos-associated diseases, including malignant mesothelioma. Including CCL3 in the standard blood tests for people at risk of asbestos exposure is likely to be of immediate and significant benefit.

Acknowledgments

This work was supported by Health and Labour Sciences Research Grants (Research on Risk of Chemical Substance 21340601, H19-kagaku-ippan-006 and H22-kagaku-ippan-005) from the Ministry of Health, Labour and Welfare, Japan and the Princess Takamatsu Cancer Research Fund H24. The authors are deeply obliged to the many healthy volunteers in Nagoya City University Graduate School of Medical Sciences for their kind provision of their own sera. We thank Ms. Akiko Iezaki for excellent secretarial assistance.

Disclosure Statement

Hiroyuki Tsuda has submitted a patent application for CCL3: #PCT/JP2012/056321. None of the other authors has a conflict of interest.

References

1 Marsili D, Comba P. Asbestos case and its current implications for global health. *Ann Ist Super Sanita* 2013; **49**: 249–51.
2 Robinson BW, Lake RA. Advances in malignant mesothelioma. *N Engl J Med* 2005; **353**: 1591–603.
3 Ramazzini C. Asbestos is still with us: repeat call for a universal ban. *Am J Ind Med* 2011; **54**: 168–73.
4 Robinson BW, Creaney J, Lake R *et al.* Mesothelin-family proteins and diagnosis of mesothelioma. *Lancet* 2003; **362**: 1612–6.
5 Maeda M, Hino O. Molecular tumor markers for asbestos-related mesothelioma: serum diagnostic markers. *Pathol Int* 2006; **56**: 649–54.
6 Hino O, Maeda M. Diagnostic tumor marker of asbestos-related mesothelioma. *Environ Health Prev Med* 2008; **13**: 71–4.
7 Iwahori K, Osaki T, Serada S *et al.* Megakaryocyte potentiating factor as a tumor marker of malignant pleural mesothelioma: evaluation in comparison with mesothelin. *Lung Cancer* 2008; **62**: 45–54.
8 Scherpereel A, Grigoriu B, Conti M *et al.* Soluble mesothelin-related peptides in the diagnosis of malignant pleural mesothelioma. *Am J Respir Crit Care Med* 2006; **173**: 1155–60.
9 Shiomi K, Hagiwara Y, Sonoue K *et al.* Sensitive and specific new enzyme-linked immunosorbent assay for N-ERC/mesothelin increases its potential as a useful serum tumor marker for mesothelioma. *Clin Cancer Res* 2008; **14**: 1431–7.
10 Rodriguez Portal JA, Rodriguez Becerra E, Rodriguez Rodriguez D *et al.* Serum levels of soluble mesothelin-related peptides in malignant and nonmalignant asbestos-related pleural disease: relation with past asbestos exposure. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 646–50.
11 Pass HI, Lott D, Lonardo F *et al.* Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. *N Engl J Med* 2005; **353**: 1564–73.
12 Amati M, Tomasetti M, Mariotti L, Tarquini LM, Valentino M, Santarelli L. Assessment of biomarkers in asbestos-exposed workers as indicators of cancer risk. *Mutat Res* 2008; **655**: 52–8.
13 Amati M, Tomasetti M, Scartozzi M *et al.* Profiling tumor-associated markers for early detection of malignant mesothelioma: an epidemiologic study. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 163–70.
14 Yasumitsu A, Tabata C, Tabata R *et al.* Clinical significance of serum vascular endothelial growth factor in malignant pleural mesothelioma. *J Thorac Oncol* 2010; **5**: 479–83.
15 Hirayama N, Tabata C, Tabata R *et al.* Pleural effusion VEGF levels as a prognostic factor of malignant pleural mesothelioma. *Respir Med* 2011; **105**: 137–42.
16 Raiko I, Sander I, Weber DG *et al.* Development of an enzyme-linked immunosorbent assay for the detection of human calretinin in plasma and serum of mesothelioma patients. *BMC Cancer* 2010; **10**: 242.
17 Yasuda M, Hanagiri T, Shigematsu Y *et al.* Identification of a tumour associated antigen in lung cancer patients with asbestos exposure. *Anticancer Res* 2010; **30**: 2631–9.
18 Maeda R, Tabata C, Tabata R, Eguchi R, Fujimori Y, Nakano T. Is serum thioredoxin-1 a useful clinical marker for malignant pleural mesothelioma? *Antioxid Redox Signal* 2011; **15**: 685–9.
19 Tabata C, Terada T, Tabata R *et al.* Serum thioredoxin-1 as a diagnostic marker for malignant peritoneal mesothelioma. *J Clin Gastroenterol* 2013; **47**: e7–11.
20 Watzka SB, Posch F, Pass HI *et al.* Detection of integrin-linked kinase in the serum of patients with malignant pleural mesothelioma. *J Thorac Cardiovasc Surg* 2011; **142**: 384–9.
21 Leivo-Korpela S, Lehtimaki L, Nieminen R *et al.* Adipokine adipsin is associated with the degree of lung fibrosis in asbestos-exposed workers. *Respir Med* 2012; **106**: 1435–40.
22 Pass HI, Levin SM, Harbut MR *et al.* Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. *N Engl J Med* 2012; **367**: 1417–27.
23 Rostila A, Puustinen A, Toljamo T *et al.* Peroxiredoxins and tropomyosins as plasma biomarkers for lung cancer and asbestos exposure. *Lung Cancer* 2012; **77**: 450–9.
24 Santarelli L, Strafella E, Staffolani S *et al.* Association of MiR-126 with soluble mesothelin-related peptides, a marker for malignant mesothelioma. *PLoS One* 2011; **6**: e18232.
25 Fujii M, Fujimoto N, Hiraki A *et al.* Aberrant DNA methylation profile in pleural fluid for differential diagnosis of malignant pleural mesothelioma. *Cancer Sci* 2012; **103**: 510–4.
26 Lehtonen H, Oksa P, Lehtimaki L *et al.* Increased alveolar nitric oxide concentration and high levels of leukotriene B(4) and 8-isoprostane in exhaled breath condensate in patients with asbestosis. *Thorax* 2007; **62**: 602–7.
27 Pelclova D, Fenclova Z, Kacer P, Kuzma M, Navratil T, Lebedova J. Increased 8-isoprostane, a marker of oxidative stress in exhaled breath condensate in subjects with asbestos exposure. *Ind Health* 2008; **46**: 484–9.
28 Chow S, Campbell C, Sandrini A, Thomas PS, Johnson AR, Yates DH. Exhaled breath condensate biomarkers in asbestos-related lung disorders. *Respir Med* 2009; **103**: 1091–7.
29 Syslova K, Kacer P, Kuzma M *et al.* Rapid and easy method for monitoring oxidative stress markers in body fluids of patients with asbestos or silica-induced lung diseases. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009; **877**: 2477–86.
30 de Gennaro G, Dragonieri S, Longobardi F *et al.* Chemical characterization of exhaled breath to differentiate between patients with malignant plueral mesothelioma from subjects with similar professional asbestos exposure. *Anal Bioanal Chem* 2010; **398**: 3043–50.
31 Lehtimaki L, Oksa P, Jarvenpaa R *et al.* Pulmonary inflammation in asbestos-exposed subjects with borderline parenchymal changes on HRCT. *Respir Med* 2010; **104**: 1042–9.
32 Dragonieri S, van der Schee MP, Massaro T *et al.* An electronic nose distinguishes exhaled breath of patients with Malignant Pleural Mesothelioma from controls. *Lung Cancer* 2012; **75**: 326–31.
33 Cristaudo A, Bonotti A, Simonini S, Bruno R, Foddis R. Soluble markers for diagnosis of malignant pleural mesothelioma. *Biomark Med* 2011; **5**: 261–73.
34 Creaney J, Yeoman D, Demelker Y *et al.* Comparison of osteopontin, megakaryocyte potentiating factor, and mesothelin proteins as markers in the serum of patients with malignant mesothelioma. *J Thorac Oncol* 2008; **3**: 851–7.
35 Grigoriu BD, Chahine B, Zerimech F *et al.* Serum mesothelin has a higher diagnostic utility than hyaluronic acid in malignant mesothelioma. *Clin Biochem* 2009; **42**: 1046–50.
36 Grigoriu BD, Grigoriu C, Chahine B, Gey T, Scherpereel A. Clinical utility of diagnostic markers for malignant pleural mesothelioma. *Monaldi Arch Chest Dis* 2009; **71**: 31–8.
37 Gube M, Taeger D, Weber DG *et al.* Performance of biomarkers SMRP, CA125, and CYFRA 21-1 as potential tumor markers for malignant mesothelioma and lung cancer in a cohort of workers formerly exposed to asbestos. *Arch Toxicol* 2011; **85**: 185–92.
38 Hollevoet K, Nackaerts K, Thimpont J *et al.* Diagnostic performance of soluble mesothelin and megakaryocyte potentiating factor in mesothelioma. *Am J Respir Crit Care Med* 2010; **181**: 620–5.
39 Luo L, Shi HZ, Liang QL, Jiang J, Qin SM, Deng JM. Diagnostic value of soluble mesothelin-related peptides for malignant mesothelioma: a meta-analysis. *Respir Med* 2010; **104**: 149–56.
40 Pass HI, Wali A, Tang N *et al.* Soluble mesothelin-related peptide level elevation in mesothelioma serum and pleural effusions. *Ann Thorac Surg* 2008; **85**: 265–72; discussion 72.
41 Ray M, Kindler HL. Malignant pleural mesothelioma: an update on biomarkers and treatment. *Chest* 2009; **136**: 888–96.

42 Tomasetti M, Santarell L. Biomarkers for early detection of malignant mesothelioma: diagnostic and therapeutic application. *Cancers (Basel)* 2010; **2**: 523–48.
43 Comabella M, Pericot I, Goertsches R *et al.* Plasma osteopontin levels in multiple sclerosis. *J Neuroimmunol* 2005; **158**: 231–9.
44 Creaney J, Yeoman D, Musk AW, de Klerk N, Skates SJ, Robinson BW. Plasma versus serum levels of osteopontin and mesothelin in patients with malignant mesothelioma–which is best? *Lung Cancer* 2011; **74**: 55–60.
45 Grigoriu BD, Scherpereel A, Devos P *et al.* Utility of osteopontin and serum mesothelin in malignant pleural mesothelioma diagnosis and prognosis assessment. *Clin Cancer Res* 2007; **13**: 2928–35.
46 Kadota J, Mizunoe S, Mito K *et al.* High plasma concentrations of osteopontin in patients with interstitial pneumonia. *Respir Med* 2005; **99**: 111–7.
47 Kariuki SN, Moore JG, Kirou KA, Crow MK, Utset TO, Niewold TB. Age- and gender-specific modulation of serum osteopontin and interferon-alpha by osteopontin genotype in systemic lupus erythematosus. *Genes Immun* 2009; **10**: 487–94.
48 Ohmori R, Momiyama Y, Taniguchi H *et al.* Plasma osteopontin levels are associated with the presence and extent of coronary artery disease. *Atherosclerosis* 2003; **170**: 333–7.
49 Xu J, Futakuchi M, Iigo M *et al.* Involvement of macrophage inflammatory protein 1alpha (MIP1alpha) in promotion of rat lung and mammary carcinogenic activity of nanoscale titanium dioxide particles administered by intrapulmonary spraying. *Carcinogenesis* 2010; **31**: 927–35.
50 Ordonez NG. The immunohistochemical diagnosis of mesothelioma: a comparative study of epithelioid mesothelioma and lung adenocarcinoma. *Am J Surg Pathol* 2003; **27**: 1031–51.
51 IARC. Asbestos (chrysotile, amosite, crocidolite, tremolite, actinolite, and anthophyllite). *IARC Monogr Arsenic, Metals, Fibres, and Dusts* 2012; **100C**: 219–309.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Study participant data.

Cancer Science
Japanese Cancer Association JCA
Open Access

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

Jiegou Xu,[1,2] David B. Alexander,[1] Masaaki Iigo,[1] Hirokazu Hamano,[3] Satoru Takahashi,[4] Takako Yokoyama,[5] Munehiro Kato,[5] Ikuji Usami,[5] Takeshi Tokuyama,[6] Masahiro Tsutsumi,[7] Mouka Tamura,[8] Tetsuya Oguri,[9] Akio Niimi,[9] Yoshimitsu Hayashi,[10] Yoshifumi Yokoyama,[10] Ken Tonegawa,[11] Katsumi Fukamachi,[12] Mitsuru Futakuchi,[12] Yuto Sakai,[12] Masumi Suzui,[12] Michihiro Kamijima,[13] Naomi Hisanaga,[14] Toyonori Omori,[15] Dai Nakae,[16] Akihiko Hirose,[17] Jun Kanno[18] and Hiroyuki Tsuda[1]

[1]Nanotoxicology Project, Nagoya City University, Nagoya, Japan; [2]Department of Immunology, College of Basic Medical Sciences, Anhui Medical University, Hefei, China; [3]Nutritional Science Institute, Morinaga Milk Industry Co., Ltd., Zama; [4]Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya; [5]Department of Respiratory Medicine, Asahi Rosai Hospital, Owariasahi; Departments of [6]Internal Medicine; [7]Pathology, Saiseikai Chuwa Hospital, Sakura; [8]Department of Internal Medicine, Nara Medical Center, National Hospital Organization, Nara; [9]Diivision of Respiratory Medicine, Allergy and Rheumatology, Nagoya City University Hospital, Nagoya; Departments of [10]Medicine; [11]Physical Medicine and Rehabilitation, Nagoya-Shi Koseiin Medical Welfare Center, Nagoya; Departments of [12]Molecular Toxicology; [13]Occupational and Environmental Health, Nagoya City University Graduate School of Medical Sciences, Nagoya; [14]Center for Campus Health and Environment, Aichi University of Education, Kariya; [15]Department of Health Care Policy and Management, Nagoya City University Graduate School of Medical Sciences, Nagoya; [16]Department of Nutritional Science and Food Safety, Faculty of Applied Biosciences, Tokyo University of Agriculture, Tokyo; Divisions of [17]Risk Assessment, Biological Safety Research Center; [18]Cellular and Molecular Toxicology, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan

Key words

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

Correspondence

Hiroyuki Tsuda, Nanotoxicology Project, Nagoya City University, 3-1 Tanabedohri, Mizuho-ku, Nagoya 467-8603, Japan.
Tel: +81-52-836-3496; Fax: +81-52-836-3497;
E-mail: htsuda@phar.nagoya-cu.ac.jp

Funding Information

Ministry of Health, Labour and Welfare, Japan. Princess Takamatsu Cancer research Fund.

Received December 15, 2014; Revised April 14, 2015; Accepted April 27, 2015

Cancer Sci **106** (2015) 825–832

doi: 10.1111/cas.12687

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

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)

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

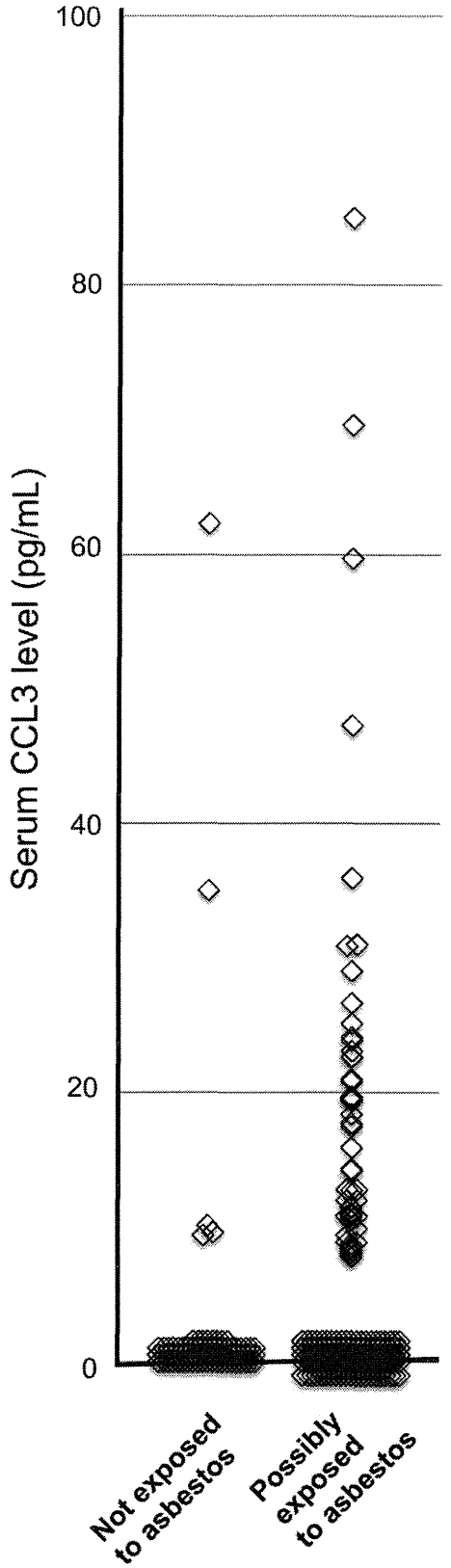

Fig. 1. Serum chemokine (C-C motif) ligand 3 (CCL3) levels in the primary study group. Levels of CCL3 in subjects not exposed to asbestos (controls) and in healthy, asymptomatic subjects possibly exposed to asbestos. Excluding the subjects without detectable CCL3, serum CCL3 levels in the group composed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

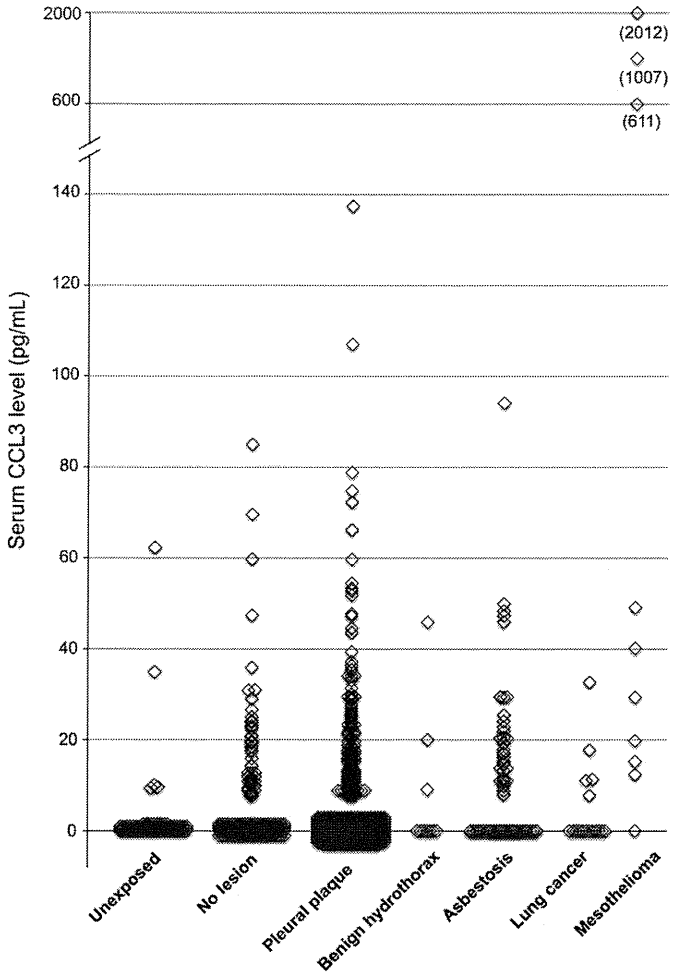

Fig. 2. Serum chemokine (C-C motif) ligand 3 (CCL3) levels in the secondary study group. Levels of CCL3 in study participants possibly exposed to asbestos and diagnosed with pleural plaque, benign hydrothorax, asbestosis, lung cancer, and mesothelioma are shown. For ease of comparison, the primary study group is shown alongside the secondary study group. The upper region of the graph is a log plot and is not continuous with the lower region of the graph. The serum CCL3 levels of the three patients plotted in the upper region are shown. Excluding the subjects without detectable CCL3 and the three subjects with exceptionally high levels of CCL3, there are no differences in CCL3 levels between any of the groups.

Table 5. Detection of serum chemokine (C-C motif) ligand 3 in the secondary study group, composed of subjects possibly exposed to asbestos and diagnosed with lung disease

Lesion category	Total number of subjects	Number of positive subjects	Detection rate, %	95% confidence interval
Exposed to asbestos				
Pleural plaque†	412	143	34.7	30.3–39.4
Asbestosis	86	34	39.5	29.9–50.1
Benign hydrothorax	10	3	30.0	10.8–60.3
Lung cancer	17	5	29.4	13.3–53.1
Malignant mesothelioma	10	9	90.0	59.6–98.2

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

tify asymptomatic persons exposed to asbestos is obviously advantageous.

The purpose of the present study was to determine whether the levels or detection rate of CCL3 in the serum were elevated in asbestos-exposed individuals. In study subjects with detectable levels of serum CCL3, the levels were not elevated in asbestos-exposed subjects compared to unexposed subjects. In contrast to serum levels, the detection rate of CCL3 in the serum of healthy, asymptomatic subjects possibly exposed to asbestos was significantly higher than in unexposed healthy subjects: the detection rate in healthy, asymptomatic subjects possibly exposed to asbestos was 30.2% and the detection rate in healthy unexposed subjects was 6.6% (see Table 3). Serum CCL3 detection rate was independent of 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.

Although the detection rate of CCL3 in the serum of healthy, asymptomatic subjects possibly exposed to asbestos was significantly higher than in the serum of unexposed control subjects, a detection rate of 30% suggests that use of serum CCL3 as a biomarker for asbestos exposure is limited. Importantly, however, verification of the presence of asbestos fibers in the lung of a healthy asymptomatic person requires microscopic examination of biopsy or autopsy specimens; therefore, confirmation of the presence of asbestos fibers in the lung or pleural tissues of a healthy, asymptomatic person possibly exposed to asbestos is not carried out. Accordingly, healthy, asymptomatic study participants with certificates of asbestos exposure must be assumed to be possibly exposed to asbestos, resulting in the asbestos-exposed no lesion study group being composed of an above average number of individuals exposed to asbestos rather than being composed entirely of asbestos-exposed individuals, and it is very unlikely that all, or even most, of the subjects in the asbestos-exposed no lesion group actually have asbestos fibers in their lungs or pleural tissues. Consequently, the true percentage of these study participants with internalized asbestos fibers having detectable levels of serum CCL3 is unknown, but it is likely to be higher, probably much higher, than 30%. Therefore, detection of CCL3 in the serum could be a valuable adjunct marker for diagnosis of asbestos exposure.

The CCL3 chemokine is produced by macrophages and other immune cells in response to infection and various other insults; therefore, obtaining multiple blood samples from a patient is needed for proper evaluation of serum CCL3 levels. It is likely that if multiple samples are obtained, the detection of serum CCL3 in healthy unexposed subjects would decrease. As detection of serum CCL3 cannot distinguish between asbestos exposure and other disease states that result in increased levels of serum CCL3, use of CCL3 as an adjunct marker of asbestos exposure is appropriate only for asymptomatic individuals.

Frustrated phagocytosis of asbestos fibers is likely to play a role in asbestos-associated diseases.[51] In addition, our previous study in rats showed that intrapulmonary dosed nanoscale titanium dioxide particles were phagocytosed by alveolar macrophages and these macrophages secreted CCL3, resulting in elevated levels of CCL3 in the serum of rats treated with titanium dioxide.[49] These data suggest that the elevation of serum CCL3 in asbestos-exposed subjects is probably due to a persistent inflammatory reaction of macrophages interacting

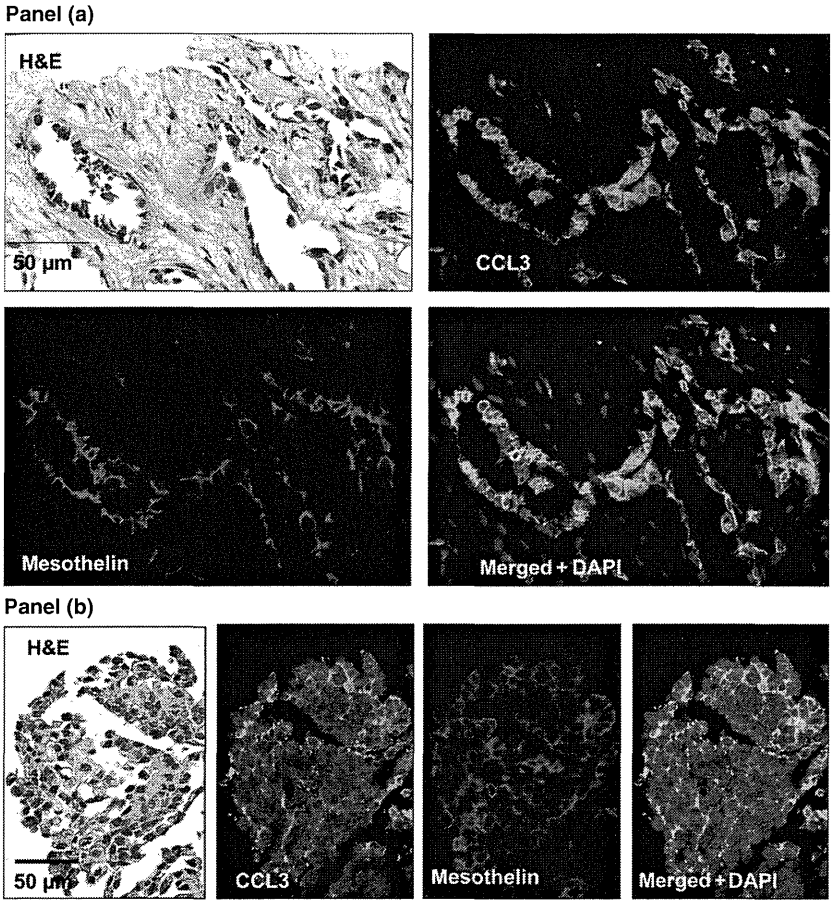

Fig. 3. Chemokine (C-C motif) ligand 3 (CCL3) and mesothelin in two biopsy specimens from patients with malignant mesothelioma. (a) Immunofluorescent staining of CCL3 and mesothelin in an enrolled biopsy case of malignant mesothelioma with glandular formation (serum CCL3, 40.2 pg/mL). Although CCL3 and mesothelin are coexpressed in the majority of tumor cells, the intracellular localization of CCL3 and mesothelin are distinct: mesothelin is localized primarily in the periphery of the tumor cells, whereas CCL3 is primarily cytoplasmic. (b) Immunofluorescent staining of CCL3 and mesothelin in an enrolled biopsy case of malignant mesothelioma with solid proliferation (serum CCL3, 2012.4 pg/mL). Similar to glandular-type malignant mesothelioma (a), most of the tumor cells expressed both CCL3 and mesothelin. Again, mesothelin is localized primarily at the cell periphery and CCL3 is more cytoplasmic.

with asbestos fibers even after the cessation of exposure to airborne asbestos.

Notably, the detection rate of CCL3 in the serum of the asbestos-exposed pleural plaque and asbestosis groups was not significantly different from the asbestos-exposed no lesion group. As the percentage of subjects diagnosed with pleural plaque and asbestosis that have asbestos fibers in their lung or pleural tissues is likely to be much higher than for the healthy, asymptomatic subjects possibly exposed to asbestos that constitute the asbestos-exposed no lesion group, this suggests that the detection rate of CCL3 in the serum of asbestos-exposed persons with certain asbestos-associated diseases is lower than for asymptomatic asbestos-exposed persons. One possible explanation for this seeming anomaly is that development of fibrotic tissue associated with inhaled asbestos fibers can prevent interaction of macrophages with the fibers.

Our study included 10 patients diagnosed with malignant mesothelioma. While this small number of malignant mesothelioma patients precludes assessment of CCL3 as a marker of malignant mesothelioma, two important observations were made. First, biopsy and autopsy specimens of malignant mesothelioma showed CCL3 expression by the tumor cells (see Fig. 3). Second, 9 of the 10 patients with malignant mesothelioma had detectable serum CCL3, and serum CCL3 levels in three of the malignant mesothelioma patients were dramatically higher than in the other study subjects. These observations suggest that expression of CCL3 by malignant mesothelioma tumor cells may be a source of the CCL3 in the serum of these patients and may result in extremely high levels of CCL3 in the serum of some malignant mesothelioma patients. We are currently accessing more malignant mesothelioma cases and appropriate controls to investigate the possibility that CCL3 may be a biomarker for malignant mesothelioma. The use of CCL3 in the pleural fluid and biopsy specimens for diagnosis of mesothelioma is also being examined.

In conclusion, inhalation of asbestos elicits a high risk of developing lung and mesothelial diseases, including fatal malignant mesothelioma. 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 important goal. The CCL3 chemokine is detectable in the serum of a significant percentage of asymptomatic persons exposed to asbestos. Persistent detection over time of CCL3 in the serum of a healthy individual possibly exposed to asbestos

can be used as an adjunct marker of asbestos exposure, and consequently as a risk marker for developing asbestos-associated diseases, including malignant mesothelioma. Including CCL3 in the standard blood tests for people at risk of asbestos exposure is likely to be of immediate and significant benefit.

Acknowledgments

This work was supported by Health and Labour Sciences Research Grants (Research on Risk of Chemical Substance 21340601, H19-kagaku-ippan-006 and H22-kagaku-ippan-005) from the Ministry of Health, Labour and Welfare, Japan and the Princess Takamatsu Cancer Research Fund H24. The authors are deeply obliged to the many healthy volunteers in Nagoya City University Graduate School of Medical Sciences for their kind provision of their own sera. We thank Ms. Akiko Iezaki for excellent secretarial assistance.

Disclosure Statement

Hiroyuki Tsuda has submitted a patent application for CCL3: #PCT/JP2012/056321. None of the other authors has a conflict of interest.

References

1 Marsili D, Comba P. Asbestos case and its current implications for global health. *Ann Ist Super Sanita* 2013; **49**: 249–51.
2 Robinson BW, Lake RA. Advances in malignant mesothelioma. *N Engl J Med* 2005; **353**: 1591–603.
3 Ramazzini C. Asbestos is still with us: repeat call for a universal ban. *Am J Ind Med* 2011; **54**: 168–73.
4 Robinson BW, Creaney J, Lake R *et al.* Mesothelin-family proteins and diagnosis of mesothelioma. *Lancet* 2003; **362**: 1612–6.
5 Maeda M, Hino O. Molecular tumor markers for asbestos-related mesothelioma: serum diagnostic markers. *Pathol Int* 2006; **56**: 649–54.
6 Hino O, Maeda M. Diagnostic tumor marker of asbestos-related mesothelioma. *Environ Health Prev Med* 2008; **13**: 71–4.
7 Iwahori K, Osaki T, Serada S *et al.* Megakaryocyte potentiating factor as a tumor marker of malignant pleural mesothelioma: evaluation in comparison with mesothelin. *Lung Cancer* 2008; **62**: 45–54.
8 Scherpereel A, Grigoriu B, Conti M *et al.* Soluble mesothelin-related peptides in the diagnosis of malignant pleural mesothelioma. *Am J Respir Crit Care Med* 2006; **173**: 1155–60.
9 Shiomi K, Hagiwara Y, Sonoue K *et al.* Sensitive and specific new enzyme-linked immunosorbent assay for N-ERC/mesothelin increases its potential as a useful serum tumor marker for mesothelioma. *Clin Cancer Res* 2008; **14**: 1431–7.
10 Rodriguez Portal JA, Rodriguez Becerra E, Rodriguez Rodriguez D *et al.* Serum levels of soluble mesothelin-related peptides in malignant and nonmalignant asbestos-related pleural disease: relation with past asbestos exposure. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 646–50.
11 Pass HI, Lott D, Lonardo F *et al.* Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. *N Engl J Med* 2005; **353**: 1564–73.
12 Amati M, Tomasetti M, Mariotti L, Tarquini LM, Valentino M, Santarelli L. Assessment of biomarkers in asbestos-exposed workers as indicators of cancer risk. *Mutat Res* 2008; **655**: 52–8.
13 Amati M, Tomasetti M, Scartozzi M *et al.* Profiling tumor-associated markers for early detection of malignant mesothelioma: an epidemiologic study. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 163–70.
14 Yasumitsu A, Tabata C, Tabata R *et al.* Clinical significance of serum vascular endothelial growth factor in malignant pleural mesothelioma. *J Thorac Oncol* 2010; **5**: 479–83.
15 Hirayama N, Tabata C, Tabata R *et al.* Pleural effusion VEGF levels as a prognostic factor of malignant pleural mesothelioma. *Respir Med* 2011; **105**: 137–42.
16 Raiko I, Sander I, Weber DG *et al.* Development of an enzyme-linked immunosorbent assay for the detection of human calretinin in plasma and serum of mesothelioma patients. *BMC Cancer* 2010; **10**: 242.
17 Yasuda M, Hanagiri T, Shigematsu Y *et al.* Identification of a tumour associated antigen in lung cancer patients with asbestos exposure. *Anticancer Res* 2010; **30**: 2631–9.
18 Maeda R, Tabata C, Tabata R, Eguchi R, Fujimori Y, Nakano T. Is serum thioredoxin-1 a useful clinical marker for malignant pleural mesothelioma? *Antioxid Redox Signal* 2011; **15**: 685–9.
19 Tabata C, Terada T, Tabata R *et al.* Serum thioredoxin-1 as a diagnostic marker for malignant peritoneal mesothelioma. *J Clin Gastroenterol* 2013; **47**: e7–11.
20 Watzka SB, Posch F, Pass HI *et al.* Detection of integrin-linked kinase in the serum of patients with malignant pleural mesothelioma. *J Thorac Cardiovasc Surg* 2011; **142**: 384–9.
21 Leivo-Korpela S, Lehtimaki L, Nieminen R *et al.* Adipokine adipsin is associated with the degree of lung fibrosis in asbestos-exposed workers. *Respir Med* 2012; **106**: 1435–40.
22 Pass HI, Levin SM, Harbut MR *et al.* Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. *N Engl J Med* 2012; **367**: 1417–27.
23 Rostila A, Puustinen A, Toljamo T *et al.* Peroxiredoxins and tropomyosins as plasma biomarkers for lung cancer and asbestos exposure. *Lung Cancer* 2012; **77**: 450–9.
24 Santarelli L, Strafella E, Staffolani S *et al.* Association of MiR-126 with soluble mesothelin-related peptides, a marker for malignant mesothelioma. *PLoS One* 2011; **6**: e18232.
25 Fujii M, Fujimoto N, Hiraki A *et al.* Aberrant DNA methylation profile in pleural fluid for differential diagnosis of malignant pleural mesothelioma. *Cancer Sci* 2012; **103**: 510–4.
26 Lehtonen H, Oksa P, Lehtimaki L *et al.* Increased alveolar nitric oxide concentration and high levels of leukotriene B(4) and 8-isoprostane in exhaled breath condensate in patients with asbestosis. *Thorax* 2007; **62**: 602–7.
27 Pelclova D, Fenclova Z, Kacer P, Kuzma M, Navratil T, Lebedova J. Increased 8-isoprostane, a marker of oxidative stress in exhaled breath condensate in subjects with asbestos exposure. *Ind Health* 2008; **46**: 484–9.
28 Chow S, Campbell C, Sandrini A, Thomas PS, Johnson AR, Yates DH. Exhaled breath condensate biomarkers in asbestos-related lung disorders. *Respir Med* 2009; **103**: 1091–7.
29 Syslova K, Kacer P, Kuzma M *et al.* Rapid and easy method for monitoring oxidative stress markers in body fluids of patients with asbestos or silica-induced lung diseases. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009; **877**: 2477–86.
30 de Gennaro G, Dragonieri S, Longobardi F *et al.* Chemical characterization of exhaled breath to differentiate between patients with malignant plueral mesothelioma from subjects with similar professional asbestos exposure. *Anal Bioanal Chem* 2010; **398**: 3043–50.
31 Lehtimaki L, Oksa P, Jarvenpaa R *et al.* Pulmonary inflammation in asbestos-exposed subjects with borderline parenchymal changes on HRCT. *Respir Med* 2010; **104**: 1042–9.
32 Dragonieri S, van der Schee MP, Massaro T *et al.* An electronic nose distinguishes exhaled breath of patients with Malignant Pleural Mesothelioma from controls. *Lung Cancer* 2012; **75**: 326–31.
33 Cristaudo A, Bonotti A, Simonini S, Bruno R, Foddis R. Soluble markers for diagnosis of malignant pleural mesothelioma. *Biomark Med* 2011; **5**: 261–73.
34 Creaney J, Yeoman D, Demelker Y *et al.* Comparison of osteopontin, megakaryocyte potentiating factor, and mesothelin proteins as markers in the serum of patients with malignant mesothelioma. *J Thorac Oncol* 2008; **3**: 851–7.
35 Grigoriu BD, Chahine B, Zerimech F *et al.* Serum mesothelin has a higher diagnostic utility than hyaluronic acid in malignant mesothelioma. *Clin Biochem* 2009; **42**: 1046–50.
36 Grigoriu BD, Grigoriu C, Chahine B, Gey T, Scherpereel A. Clinical utility of diagnostic markers for malignant pleural mesothelioma. *Monaldi Arch Chest Dis* 2009; **71**: 31–8.
37 Gube M, Taeger D, Weber DG *et al.* Performance of biomarkers SMRP, CA125, and CYFRA 21-1 as potential tumor markers for malignant mesothelioma and lung cancer in a cohort of workers formerly exposed to asbestos. *Arch Toxicol* 2011; **85**: 185–92.
38 Hollevoet K, Nackaerts K, Thimpont J *et al.* Diagnostic performance of soluble mesothelin and megakaryocyte potentiating factor in mesothelioma. *Am J Respir Crit Care Med* 2010; **181**: 620–5.
39 Luo L, Shi HZ, Liang QL, Jiang J, Qin SM, Deng JM. Diagnostic value of soluble mesothelin-related peptides for malignant mesothelioma: a meta-analysis. *Respir Med* 2010; **104**: 149–56.
40 Pass HI, Wali A, Tang N *et al.* Soluble mesothelin-related peptide level elevation in mesothelioma serum and pleural effusions. *Ann Thorac Surg* 2008; **85**: 265–72; discussion 72.
41 Ray M, Kindler HL. Malignant pleural mesothelioma: an update on biomarkers and treatment. *Chest* 2009; **136**: 888–96.

42 Tomasetti M, Santarell L. Biomarkers for early detection of malignant mesothelioma: diagnostic and therapeutic application. *Cancers (Basel)* 2010; **2**: 523–48.
43 Comabella M, Pericot I, Goertsches R *et al.* Plasma osteopontin levels in multiple sclerosis. *J Neuroimmunol* 2005; **158**: 231–9.
44 Creaney J, Yeoman D, Musk AW, de Klerk N, Skates SJ, Robinson BW. Plasma versus serum levels of osteopontin and mesothelin in patients with malignant mesothelioma–which is best? *Lung Cancer* 2011; **74**: 55–60.
45 Grigoriu BD, Scherpereel A, Devos P *et al.* Utility of osteopontin and serum mesothelin in malignant pleural mesothelioma diagnosis and prognosis assessment. *Clin Cancer Res* 2007; **13**: 2928–35.
46 Kadota J, Mizunoe S, Mito K *et al.* High plasma concentrations of osteopontin in patients with interstitial pneumonia. *Respir Med* 2005; **99**: 111–7.
47 Kariuki SN, Moore JG, Kirou KA, Crow MK, Utset TO, Niewold TB. Age- and gender-specific modulation of serum osteopontin and interferon-alpha by osteopontin genotype in systemic lupus erythematosus. *Genes Immun* 2009; **10**: 487–94.
48 Ohmori R, Momiyama Y, Taniguchi H *et al.* Plasma osteopontin levels are associated with the presence and extent of coronary artery disease. *Atherosclerosis* 2003; **170**: 333–7.
49 Xu J, Futakuchi M, Iigo M *et al.* Involvement of macrophage inflammatory protein 1alpha (MIP1alpha) in promotion of rat lung and mammary carcinogenic activity of nanoscale titanium dioxide particles administered by intrapulmonary spraying. *Carcinogenesis* 2010; **31**: 927–35.
50 Ordonez NG. The immunohistochemical diagnosis of mesothelioma: a comparative study of epithelioid mesothelioma and lung adenocarcinoma. *Am J Surg Pathol* 2003; **27**: 1031–51.
51 IARC. Asbestos (chrysotile, amosite, crocidolite, tremolite, actinolite, and anthophyllite). *IARC Monogr Arsenic, Metals, Fibres, and Dusts* 2012; **100C**: 219–309.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Study participant data.

Biochemical and Biophysical Research Communications 446 (2014) 702–708

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Review

Implications of cholesterol autoxidation products in the pathogenesis of inflammatory diseases

Noriyuki Miyoshi [a,*], Luigi Iuliano [b,*], Susumu Tomono [a], Hiroshi Ohshima [a]

[a] Laboratory of Biochemistry, Graduate School of Integrated Pharmaceutical and Nutritional Sciences, Graduate Program in Food and Nutritional Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

[b] Department of Medico-Surgical Sciences and Biotechnologies, Laboratory of Vascular Biology and Mass Spectrometry, Sapienza University of Rome, Latina 04100, Italy

ARTICLE INFO

Article history:
Available online 9 January 2014

Keywords:
Cholesterol autoxidation
Inflammation
Oxidative stress
Oxysterols
Secosterol

ABSTRACT

There is rising interest in non-enzymatic cholesterol oxidation because the resulting oxysterols have biological activity and can be used as non-invasive markers of oxidative stress *in vivo*. The preferential site of oxidation of cholesterol by highly reactive species is at C_7 having a relatively weak carbon–hydrogen bond. Cholesterol autoxidation is known to proceed via two distinct pathways, a free radical pathway driven by a chain reaction mechanism (type I autoxidation) and a non-free radical pathway (type II autoxidation). Oxysterols arising from type II autoxidation of cholesterol have no enzymatic correlates, and singlet oxygen ($^1\Delta gO_2$) and ozone (O_3) are the non-radical molecules involved in the mechanism. Four primary derivatives are possible in the reaction of cholesterol with singlet oxygen via ene addition and the formation of 5α-, 5β-, 6α- and 6β-hydroxycholesterol preceded by their respective hydroperoxyde intermediates. The reaction of ozone with cholesterol is very fast and gives rise to a complex array of oxysterols. The site of the initial ozone reaction is at the $\Delta_{5,6}$ –double bond and yields 1,2,3-trioxolane, a compound that rapidly decomposes into a series of unstable intermediates and end products. The downstream product 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (sec-A, also called 5,6-secosterol), resulting from cleavage of the B ring, and its aldolization product (sec-B) have been proposed as a specific marker of ozone-associated tissue damage and ozone production *in vivo*. The relevance of specific ozone-modified cholesterol products is, however, hampered by the fact sec-A and sec-B can also arise from singlet oxygen via Hock cleavage of 5α-hydroperoxycholesterol or via a dioxietane intermediate. Whatever the mechanism may be, sec-A and sec-B have no enzymatic route of production *in vivo* and are reportedly bioactive, rendering them attractive biomarkers to elucidate oxidative stress-associated pathophysiological pathways and to develop pharmacological agents.

Contents

1. Introduction 703
2. Cholesterol autoxidation 703
3. Cholesterol aldehydes: ozone or not ozone? 703
4. Biological activity of secosterols 704
5. *In vivo* detection 705
Acknowledgments 706
References 706

Abbreviations: Aβ, amyloid-β; Chol-OOHs, cholesterol hydroperoxides; C27 3β -HSD, 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase; DNPH, dinitrophenyl hydrazine; DH, dansyl hydrazine; GP, Girard P; GC/MS, gas chromatography/mass spectrometry; HMP, 2-hydrazino-1-methylpyridine; LC/MS, liquid chromatography/mass spectrometry; LOD, limit of detection; LOO$^\cdot$, lipid peroxyl radicals; LO$^\cdot$, lipid alkoxyl radicals; MBP, myelin basic protein; MPO, myeloperoxidase; PBH, pyrenebutyric hydrazine; PHGPx, phospholipid-hydroperoxide glutathione peroxidase; sec-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secA-COOH, 3β-hydroxy-5-oxo-secocholestan-6-oic acid; sec-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde; secB-COOH, 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid; 5α-Chol-OOH, 5α-cholesterol-hydroperoxide; 5β-Chol-OOH, 5β-cholesterol-hydroperoxide; 6α-Chol-OOH, 6α-cholesterol-hydroperoxide; 6β-Chol-OOH, 6β-cholesterol-hydroperoxide; 7α-OHC, 7α-hydroxycholesterol; 7α-Chol-OOH, 7α-cholesterol-hydroperoxide; 7β-Chol-OOH, 7β-cholesterol-hydroperoxide; 24-OHC, 24-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol.

* Corresponding authors. Fax: +81 54 264 5530 (N. Miyoshi). Fax: +39 06 62 29 1089 (L. Iuliano).
E-mail addresses: miyoshin@u-shizuoka-ken.ac.jp (N. Miyoshi), luigi.iuliano@uniroma1.it (L. Iuliano).

0006-291X/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.107

1. Introduction

Oxysterols are derivatives of cholesterol containing one or more oxygen atoms, other than the OH group on C_3, as hydroxyl, keto, epoxide or peroxyde group – that is mounted on the A and B ring or on the side chain. Oxysterols can be generated either enzymatically, mainly by the group of cytochrome (CYP) P450 family, or by autoxidation [1]. In brief, in biological systems oxygenation on side-chain is almost exclusively enzymatic, while that on the A and B ring can occur both enzymatically and by autoxidation.

Oxysterols arising from enzymatic synthesis can be used as markers of their respective cytochrome activity. Circulating 7α-hydroxycholesterol (7α-OHC), a starting intermediate in the biosynthesis of bile acids [2], correlates with the activity of CYP7A1 [3], 7α-hydroxy-4-cholesten-3-one, a conversion product of 7α-OHC is formed by the microsomal 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (C27 3β-HSD) [4], 4β-hydroxycholesterol can be used as an endogenous marker of CYP3A4 and CYP3A5 activity [5], 24S-hydroxycholesterol (24-OHC) is the product of the brain-specific cholesterol 24-hydroxylase (CYP46A1) [6,7], 27-hydroxycholesterol (27-OHC) is formed by the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1), which is widely distributed in tissues [8,9]. Examples of oxysterols forming enzymes different than the cyt450 family are cholesterol 25-hydroxylase [10] and oxidosqualene cyclase [11], which produce 25-hydroxycholesterol and 24(S),25-epoxycholesterol, respectively, and cholesterol epoxide hydrolase that converts 5,6-epoxydes into cholesterol-triol [12].

The susceptibility of cholesterol to non-enzymatic oxidation has generated considerable interest in oxysterols as potential markers for the non-invasive study of oxidative stress *in vivo*. Additional interest in oxysterols stems from the biological activity of many oxysterols that is useful to elucidate pathophysiological pathways in human diseases and for pharmacological purposes [13]. Cholesterol autoxidation proceeds via two distinct pathways, a free radical pathway driven by a chain reaction mechanism (type I) and a non-free radical pathway (type II), which is driven stoichiometrically by highly reactive oxygen species [13,14]. The unique cholesterol double bond between carbons 5 and 6 is the most vulnerable site for oxidation by free radicals and highly reactive species [15].

2. Cholesterol autoxidation

Type I autoxidation involves initiation and propagation reactions. Free radicals provide the initiation step by hydrogen abstraction, formation of a carbon centered radical and subsequent oxygen capture. Afterwards, the process advances through free radical intermediates – including, peroxyl radicals ($LOO^{\cdot}$) and alkoxyl radicals ($LO^{\cdot}$) – that in turn recruit additional non-oxidized molecules and provoke the spreading of the process via a chain-reaction, the propagation phase.

Despite the hydrogen bond-dissociation energy of C_7-cholesterol is higher than the hemolytic cleavage of allylic hydrogens in polyunsaturated fatty acids [16], entropic factors determine a predominant role of cholesterol oxidation in cellular membranes [17].

A multitude of oxysterols can be formed upon type I autoxidation, but analytical issues restrain the number of species usable as markers of oxidative stress in biological matrices. The species that actually perform well on GC/MS, which is the gold standard for oxysterols measurement, are: 4α- and 7β-hydoxycholesterol, 5α,6α- and 5β,6β-epoxides, and 7-ketocholesterol [13]. Recent studies from Porter and co-workers have established the product distribution of several oxysterols obtained through the free radical chain oxidation of the cholesterol precursor 7-dehydrocholesterol [18].

In type II autoxidation the main molecules that are involved in cholesterol oxidation are the non-radical species singlet oxygen and ozone. Singlet oxygen is formed by an input of energy, such as photoactivation, the Russell mechanism, based on the decomposition of lipid hydroperoxides, and by the reactions of hypochlorous acid and hydrogen peroxide. The following primary species are possible in the reaction of cholesterol with singlet oxygen via ene addition: 5α-cholesterol-hydroperoxide (5α-Chol-OOH), 5β-cholesterol-hydroperoxide (5β-Chol-OOH), 6α-cholesterol-hydroperoxide (6α-Chol-OOH), 6β-cholesterol-hydroperoxide (6β-Chol-OOH), and Chol-1,2-dioxetane. The formation of 5α-Chol-OOH is highly favored at a rate of approximately one order of magnitude higher than that of 6α-Chol-OOH and 6β-Chol-OOH [19]. Minor products of ozone-driven cholesterol oxidation are 5α,6α- and 5β,6β-epoxides, which have been found to form in ethyl acetate [20], but their participation in a physiological environment is not reported. The 7α- and 7β-Chol-OOH are formed during the reaction of singlet oxygen with cholesterol and generated indirectly by the allylic rearrangement of 5α-Chol-OOH [21], which takes place at high peroxidation levels but is negligible under limited cholesterol oxidation (<5%) [22]. Cholesterol hydroperoxides are susceptible to 1 e^- reduction that gives rise to alkoxyl- and peroxyl-radical intermediates that, in turn, can trigger chain reactions and amplify the free radical cascade of cholesterol oxidation and the oxidative damage. All cholesterol hydroperoxides are expected to be equally susceptible to 1 e^- reduction in the presence of metal catalysts. Similar rate constants have been reported for the reduction of 5α-Chol-OOH and 6α-Chol-OOH formation during incubation with an iron-based redox cycling system in a homogeneous solution in which cholesterol was the only chain-carrying species [19]. The potency of 5α-Chol-OOH and 7α-Chol-OOH as chain initiators is comparable [23]. Cholesterol hydroperoxides (Chol-OOHs) are resistant to direct 2 e^- reduction that is catalyzed by Se-dependent glutathione peroxidase [24]. This means that Chol-OOHs have a potential long half-life in cells. The only enzyme capable of catalyzing the reduction of Chol-OOHs to stable diols, is the phospholipid-hydroperoxide glutathione peroxidase (PHGPx) [25]. However, the reduction of Chol-OOH by PHGPx is 6 times slower compared to the reduction of phospholipid hydroperoxides [26], and shows different rate constants ranging from $0.8 \times 10^2\ min^{-1}$ for 5α-Chol-OOH to $\approx 6 \times 10^2\ min^{-1}$ for 6α-Chol-OOH and 6β-Chol-OOH [19]. Thus, 5α-Chol-OOH results the most abundant product of singlet oxygen reaction with cholesterol, and the least resistant to detoxification via PHGPx. The forward products arising from type-II cholesterol autoxidation are cholesterol aldehydes.

3. Cholesterol aldehydes: ozone or not ozone?

3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (sec-A), the major cholesterol ozonolysis products [20], is unstable in physiological aqueous conditions, such as culture medium containing serum, and is readily converted to its aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (sec-B) (Fig. 1) [27]. In part, sec-A and sec-B are further converted to their oxidized forms 3β-hydroxy-5-oxo-secocholestan-6-oic acid (secA-COOH) and 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid (secB-COOH) in culture media and probably *in vivo* [27]. Recently, ozonolysis products of the major cholesteryl fatty acid esters transported in human LDL have been reported [28]. Under a flux of ozone, cholesteryl palmitate gives rise to palmitoyl-sec-A and palmitoyl-sec-B. Instead, ozonolysis of cholesterol esterified with unsaturated fatty acids oleate and linoleate admits the initial