Original Article

Elemental analysis of inorganic dusts in lung tissues of interstitial pneumonias

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People have the opportunity to inhale inorganic dusts under various environments. Inorganic dust exposures as a result of occupational exposure may induce or modulate pulmonary fibrosis. We analyzed the deposition of elements in lung tissues of patients with idiopathic pulmonary fibrosis (IPF) and compared element deposition with chronic hypersensitivity pneumonitis (chronic HP) and collagen vascular diseases (CVD).

Thirty-five patients (18 men and 17 women with the mean age of 64.3) were studied, including 15 IPF, 8 chronic HP, 6 CVD, and 6 control patients. Four IPF patients have occupational dust exposures. Inorganic particles were counted by polarizing light microscopy and scanning electron microscopy. Energy dispersive X-ray spectroscopy was performed to analyze an elemental deposition.

The number of birefringent particles was greater in IPF, even in IPF without occupational exposure, than in controls. The silicon (Si) / sulfur (S) ratio and aluminium (Al) / S ratio were increased in IPF independent of occupational exposure. A point elemental analysis showed that the major compound of the particles was aluminium-silicate in IPF.

These results suggest that unrecognized dust exposures are relatively common in some IPF patients and aluminium-silicate could be associated with the disease process of IPF.

Key words: elemental analysis, energy dispersive X-ray spectroscopy, idiopathic pulmonary fibrosis, interstitial pneumonia, scanning electron microscopy

Introduction

Inorganic dusts such as silica, silicates, asbestos, aluminium and cobalt could be the cause of pulmonary fibrosis as a result of occupational exposure.[1-3] Furthermore, people have the opportunity to inhale inorganic dusts under various environments and these unrecognized dust exposures are likely to be more frequent than we anticipate, and may lead to the development of pulmonary diseases including interstitial pneumonias (IPs).

Many acute and chronic lung disorders with pulmonary inflammation and fibrosis are collectively referred to as interstitial lung diseases. Idiopathic pulmonary fibrosis (IPF) is one of several idiopathic interstitial pneumonias and its etiology is unknown. IPF is a progressive and usually fatal lung disease characterized by irreversible distortion of the lung's architecture. Several factors such as smoking, drugs and viral

infection, have been presumed to be related to the pathogenesis of IPF.[4] Epidemiologically, death due to IPF was reported to increase in the traditionally industrialized areas[5] and environmental triggers including dust exposures have been reported as important factors in the etiology of IPF.[6]

The international consensus statement of the American Thoracic Society (ATS) and European Respiratory Society (ERS) described that other known causes of interstitial lung disease, such as drug toxicities, environmental exposures, and collagen vascular diseases (CVD), should be excluded for the diagnosis of IPF. Metal and wood dust exposures are listed as risk factors for developing pulmonary fibrosis.[4] On the basis of this current description, we are not certain whether we should regard inorganic-dust exposures in IPF as the direct cause or as an ancillary risk factor of pulmonary fibrosis.

Previous studies have employed the elemental analyses of tissues from IPF patients. MONSO *et al* have reported that a part of IPF lungs contained innumerable asbestos fibers, and the silicon (Si) / sulfur (S) ratio in IPF lung tissue was significantly greater than that in normal lung.[7,8] Since these studies were performed before the announcement of the consensus statement, there might be differences in the present methods of diagnosing IPF. Recently, an epidemiologic study in Japan has demonstrated that exposures to metal dust are significant risk factors of IPF even in the cases diagnosed by recent criteria.[9] Therefore, it is essential to examine which metals are prominent factors in the pathogenesis of IPF.

Hypersensitivity pneumonitis (HP) is an immunologically induced lung disease in response to inhaled exposure to organic dusts[10,11] and bird fancier's lung (BFL) is one of the HPs caused by inhalation of avian antigens.[12] Patients with BFL tend to develop chronic HP usually induced by prolonged exposures to a small quantity of bird antigens[11] and some of which are insidious showing radiologic, histologic features of usual interstitial pneumonia (UIP) pattern. The UIP pattern is a typical histological finding of IPF. CVD also sometimes develop IPs, which frequently show pathological patterns of nonspecific interstitial pneumonia (NSIP) and sometimes exhibit UIP.[13] These IPs occasionally show strikingly similar presentations to IPF. Accordingly, it is possible that these IPs could be modified by unrecognized dust exposures.

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) have been generally effective to detect elemental distribution in various materials and tissues, and have been used in the examination of dental implants, artificial joints[14] and lung tissues from patients with occupational dust exposures.[15,16] In the present study, we analyzed an elemental deposition in lung tissues of IPF patients diagnosed based on the recent definition, and compared this to other IPs.

Materials and Methods

Study population

We analyzed an elemental composition of inorganic deposit in lung tissues of 35 patients diagnosed at two hospitals in Tokyo and Seto City (18 men and 17 women with the mean age of 64.3). Twenty-eight samples out of 35 subjects were obtained by video-assisted thoracoscopic surgery and 7 of them by autopsy during the period from January 1991 to December 2003. Six control patients with other pulmonary diseases who do not have IPs (3 lung cancer, 2 metastatic lung tumor and 1 emphysema), 6 patients with CVD (2 rheumatoid arthritis, 1 scleroderma, 1 mixed connective tissue disease, 1 Sjögren's syndrome and 1 scleroderma with Sjögren's syndrome), 8 with chronic BFL (cBFL) and 15 with IPF were studied. Background characteristics of all patients were evaluated by detailed medical interviews, such as smoking history, occupational history, a history of exposures to birds and a history of environmental exposures to dusts. In the present study, all IPF subjects were diagnosed by revaluating pathological and radiological findings based on the recent diagnostic criteria in the international consensus statement.[4] Four patients with IPF lived at Seto City which is well-known for producing chinawares. They proved to have a history of initially unrecognized occupational dust exposure, such as (1)crushing stones for 35 years, (2)making chinawares for 30 years, (3)making chinawares for 5 years, and (4)carrying sands and soils for 35 years. Criteria for the diagnosis of chronic HP were the same as those employed in studies reported by Yoshizawa *et al.*[11,12] Informed consent was obtained from all patients.

Detection of birefringent particles by polarizing light microscopy

The number of birefringent particles in lung tissues was counted by polarizing light microscopy (Fig 1). Each counting was performed randomly in the total 10 fields including 5 fields at the area around bron-
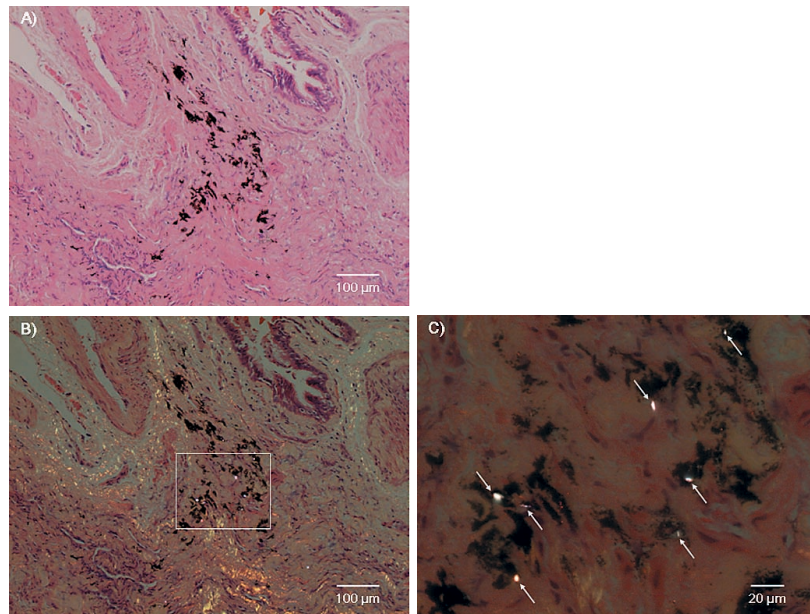
Fig. 1. (A) Video-assisted thoracoscopic lung biopsy specimen of chronic HP patient (hematoxylin and eosin stain). (B) Polarizing light microscopy image of Figure 1(A). Inorganic dusts were observed as birefringent substances. (C) A higher magnification image of the indicated area of Figure 1(B) shows birefringent inorganic particles deposited in lung tissue (arrows).

chovascular bundle and 5 fields at the area of fibrotic lung in IP subjects, or the area of alveolar wall in control subjects.

Elemental analysis by SEM and EDS

Paraffin-embedded lung sections were cut in 4 μm thick and deparaffined and dehydrated. These sections were placed on the carbon discs and spatter-coated using osmium plasma coater (NL-OPC80N; Filgen Inc.; Nagoya, Japan). Sections were examined by SEM (S-4500; Hitachi Ltd.; Hitachinaka, Japan). Images of mineral dusts were obtained by using lower detector and elemental analyses were performed by EDS (EMAX-7000; Horiba Ltd.; Kyoto, Japan). Acquisition time of the EDS spectrum was 100sec at 20kV of acceleration voltage. The EDS spectrum was quantitatively assessed according to the intensity of each spectrum (Fig 2). The levels of each element were measured as elemental weights and expressed as the ratio to sulfur (S) which might depend on the amount of tissue. As it was comprehended that birefringent particles were located mainly at anthracotic areas in the above-mentioned polarizing light microscopy analysis, anthracotic areas were randomly selected by SEM and assigned to EDS. The quantity of elemental deposition was measured in randomly selected 5 fields at ×1000
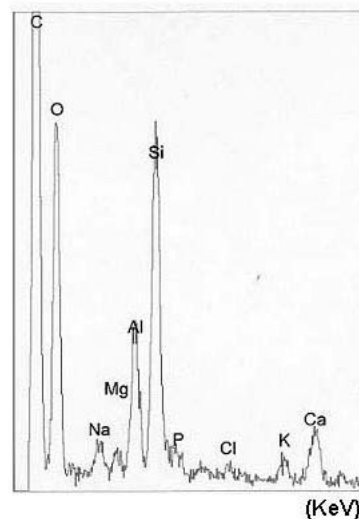

Fig. 2. Representative data by EDS (energy dispersive X-ray spectroscopy).

magnification and these values were averaged.

The high magnification analysis by SEM

Furthermore, the high magnification analysis was performed to count the number of inorganic particles sensitively and to identify the kind of particles. This

analysis was based on the method described by ABRAHAM et *al*, with a slight modification.[16] We counted the number of particles through secondary SEM image in randomly selected total 30 fields at $\times$ 3000 magnification (1259 μm^2 per field). All particles were analyzed by using a point elemental analysis and divided into four kinds of compound, that is silica (SiO_2), aluminium-silicate (compound containing silica bound to aluminium and other cations), talc ($Mg_3Si_4O_{10}(OH)_2$) and other metal. We also used a linear elemental analysis and an elemental mapping analysis to confirm the distribution of elements.

Statistical analysis

Comparisons of patient characteristics among five groups were made with the chi-square test. Data are expressed as mean $\pm$SE. Differences between the two groups were evaluated using the Mann-Whitney U test, whereas comparisons of data among five groups were assessed by the Kruskal-Wallis test followed by the Steel-Dwass test. Differences were considered statistically significant when p<0.05. The statistical analyses were performed using the software Statcel statistical analysis system.

Results

Baseline characteristics of the patients in this study are shown in Table 1. No significant differences were observed in age, gender and smoking history among five groups. All patients with cBFL, 1 with CVD, and 1 with IPF had a history of exposures to birds. And detailed medical interviews revealed that all patients, except for 4 with IPF, had no history of recognized dust or metal exposures. A Brinkman Index was calculated for quantitative estimation of smoking history, in which the number of cigarettes smoked per day was multiplied by the years of smoking. The Brinkman Index showed no significant differences among five groups. All patients with IPF, 4 with CVD, 7 with cBFL showed a histological pattern of UIP.

The number of birefringent particles

Differences of the total number of birefringent particles among five groups were statistically significant (p=0.0039 by Kruskal-Wallis test). The total number of birefringent particles in IPF without occupational exposure was greater as compared to that in control and CVD (44.6$\pm$8.4 VS 15.3$\pm$3.1 VS 21.3$\pm$2.3; p<0.05, p<0.05, respectively) (Fig 3). However, differences of the total number of birefringent particles between in IPF with occupational exposure and in other groups were not statistically significant. In all groups except for IPF with occupational exposure, the number of birefringent particles in the area around bron-
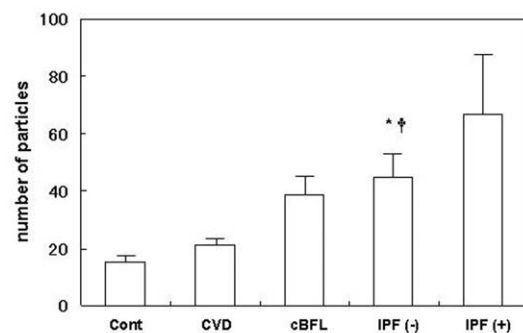

Fig. 3. The number of birefringent particles detected in the total 10 fields by polarizing light microscopy. Results are expressed as mean $\pm$SE. IPF (-) = IPF without occupational exposure; IPF (+) = IPF with occupational exposure which was later indicated after precise medical interview.
*p < 0.05 versus Cont; † p < 0.05 versus CVD.

Table 1. Baseline characteristics of the patients.

	Cont (n = 6)	CVD (n = 6)	cBFL (n = 8)	IPF(-) (n = 11)	IPF(+) (n = 4)
Age (yr)	65.5$\pm$2.3	56.3$\pm$4.7	63.9$\pm$3.6	69.2$\pm$2.7	61.8$\pm$2.5
Sex (M/F)	2/4	1/5	4/4	8/3	3/1
Smoker / Nonsmoker	3/3	1/5	3/5	7/4	2/2
Brinkman Index	478.3$\pm$221.8	358.3$\pm$358.3	412.5$\pm$203.9	537.3$\pm$190.0	372.5$\pm$281.5
Pathological finding (U:UIP , N:NSIP)		U:4, N:2	U:7, N:1	U:11, N:0	U:4, N:0

Definition of abbreviations: Cont = control subjects; CVD = collagen vascular diseases; cBFL = chronic bird fancier's lung; IPF (-) = idiopathic pulmonary fibrosis without occupational exposure; IPF (+) = IPF with occupational exposure which was later indicated after precise medical interview; Brinkman Index = the number of cigarettes smoked per day multiplied by the years of smoking; UIP = usual interstitial pneumonia; NSIP = nonspecific interstitial pneumonia.

chovascular bundle was greater significantly than that in the area of fibrotic lung (Fig 4).

Elemental analysis at low magnification

There were significant differences in the Si/S and aluminium(Al)/S ratio among five groups (p=0.0018, p=0.0012, respectively, by Kruskal-Wallis test). In IPF with occupational exposure, increased Si/S ratio was

detected more than in control and cBFL (3.1 ± 0.7 VS 0.4 ± 0.2 VS 1.0 ± 0.2; p<0.05, p<0.05, respectively) (Fig 5A) and Al/S ratio was detected more than in control, CVD and cBFL (1.4 ± 0.2 VS 0.3 ± 0.1 VS 0.6 ± 0.1 VS 0.5 ± 0.1; p<0.05, p<0.05, p<0.05, respectively) (Fig 5B). Moreover, also in IPF without occupational exposure, Si/S ratio was larger than in control (1.8 ± 0.3 VS 0.4 ± 0.2; p<0.05) (Fig 5A) and Al/S ratio was larger than in control (0.8 ± 0.1 VS 0.3 ± 0.1; p<0.05) (Fig 5B). No significant differences were shown in the quantity of elements except Si and Al (Fig 5C, D).

Elemental analysis at high magnification

More detailed analysis was performed to identify the total number and the kind of elements including Si and/or Al (Fig 6). There were significant differences in the number of total particles and aluminium-silicate particles detected by SEM among five groups (p=0.0035, p=0.0029, respectively, by Kruskal-Wallis test). The total number of particles in IPF with occupational exposure was greater than that in CVD (p<0.05 ; Table 2). Even in IPF without occupational exposure, the total number of particles was greater than that in CVD (p<0.05). The kind of particles was analyzed by EDS and divided into four groups (Fig 7A-D). The number of particles as aluminium-silicate was greater in IPF with occupational exposure than in CVD (p<0.05). In
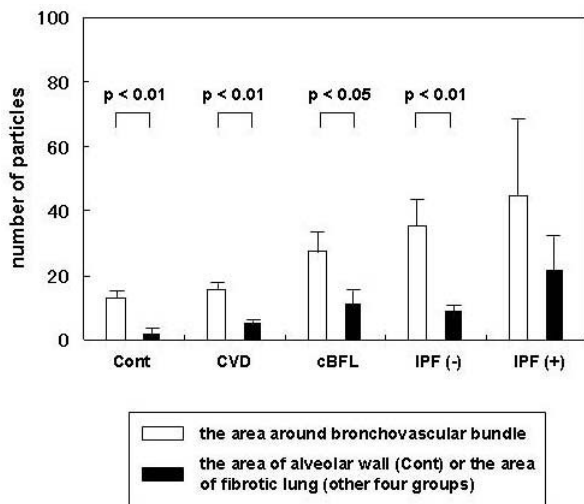

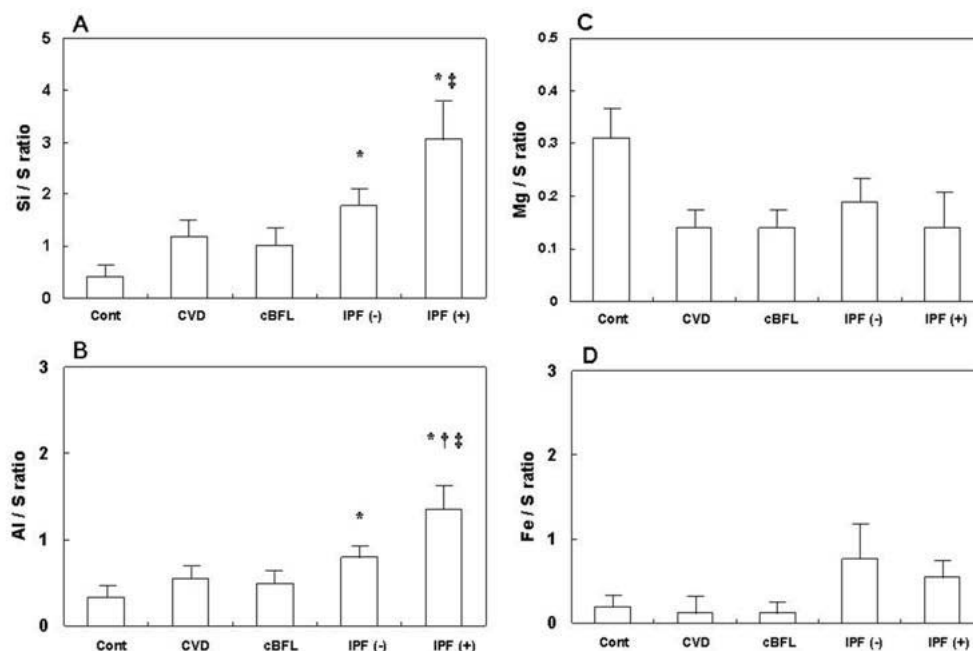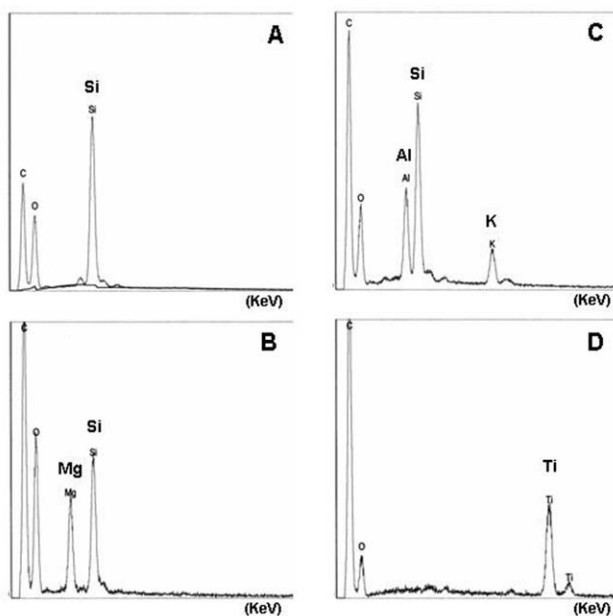**Fig. 4.** Comparisons of the number of birefringent particles between in 5 fields at the area around bronchovascular bundle and in 5 fields at the area of alveolar wall (Cont) or the area of fibrotic lung (other four groups). Results are expressed as mean $\pm$SE.
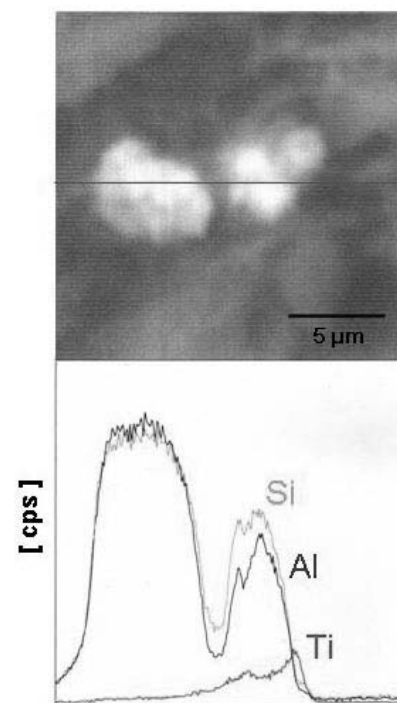

Fig. 5. The quantity of elemental deposition of silicon (Si) (A), aluminium (Al) (B), magnesium (Mg) (C) and iron (Fe) (D). The amount is expressed as the ratio to sulfur (S).
*p < 0.05 versus Cont; †p < 0.05 versus CVD; ‡p < 0.05 versus cBFL.

Table 2. The number of particles detected by SEM.

	Cont (n = 6)	CVD (n = 6)	cBFL (n = 8)	IPF(-) (n = 11)	IPF(+) (n = 4)
Total particles	7.7±1.9	4.8±1.1	9.6±2.9	32.7±8.3 †	126.0±75.5 †
Silica	0.17±0.17	0.50±0.22	0.13±0.13	0.91±0.31	8.0±3.4
Aluminium-silicate	6.8±1.7	3.5±1.0	8.8±2.8	30.7±8.2 †	115.5±73.4 †
Talc	0.33±0.21	0.17±0.17	0.75±0.41	0.27±0.19	0.5±0.5
Other metal	0.33±0.21	0.67±0.33	0	0.82±0.33	1.8±1.0

† p<0.05 versus CVD.
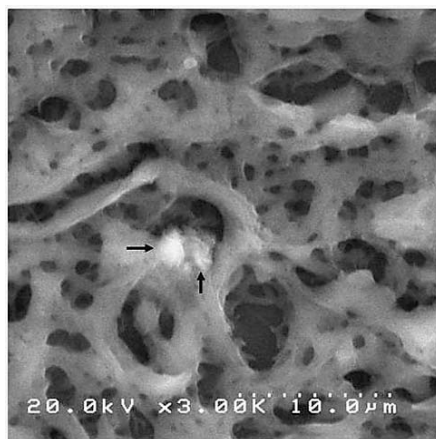
Fig. 6. High magnification image of IPF lung tissue by SEM (×3000). The particles of aluminium-silicate are shown (arrows).

Fig. 8. Top: Inorganic particles observed by SEM in IPF subject. Bottom: Linear analysis of the area indicated by the horizontal line in the top image using EDS. The full scale of three elements is the same level (= 2500 cps). Height of the line indicates the distribution and the relative quantity of each element in the particle.

Fig. 7. Examples of each EDS spectrum of four compounds, silica (A), aluminium-silicate (B), talc (C) and metal (titanium) (D).

addition, in IPF without occupational exposure, the number of particles of aluminium-silicate was greater than in CVD (p<0.05). The number of other compounds as silica, talc and other metals showed no significant differences among five groups (Table 2).

Linear analysis and elemental mapping analysis

We could detect the distribution of several elements simultaneously by using a linear analysis and a mapping analysis. We confirmed that silica contained only Si and aluminium-silicate contained Si, Al and other
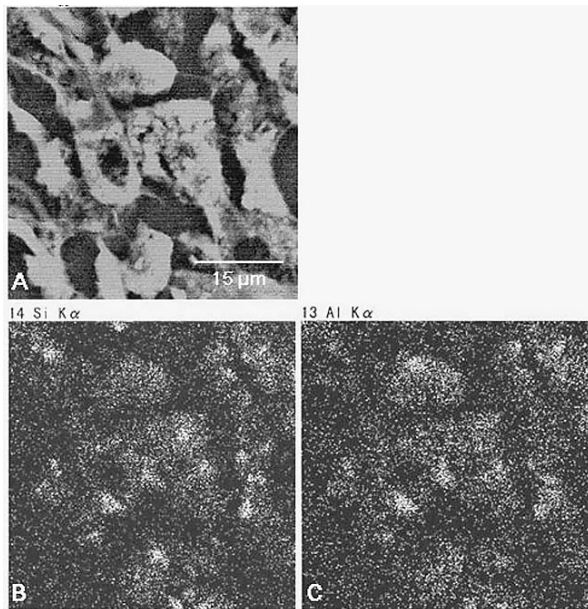
Fig. 9. Elemental mapping analysis by SEM in IPF subject. The high magnification observation by SEM (A). The elemental distribution in lung tissue of silicon (B) and aluminium (C) is shown.

cations together in analyzed particles. These elements were not detected in the area outside of particles (Fig 8, 9).

Discussion

In the present study, we demonstrated that the number of inorganic particles in lung tissues was greater even in IPF without occupational exposure than that in control or in other IPs. Two elements, Si and Al, were detected more in lung tissues of IPF both with and without occupational exposure than that in other groups. From detailed SEM analyses, most of these elements originated from compounds identified as aluminium-silicate.

Polarizing light microscopy is a good preliminary method for detecting various minerals in routine histologic slides and gives useful information in diagnosing subjects of pneumoconiosis.[17] The accuracy of this method to identify silica has been reported to be equivalent to that of SEM[17] and silicates can also be detected as strong birefringent particles under polarizing light microscopy.[18] We analyzed the lung tissues of chronic HP, CVD and IPF as well as control subjects by this method, and reconfirmed the description of past reports concerning the detectability of this method.[17,18]

The number of inorganic particles in lung tissues was greater and the deposition of Si and Al was detected more in IPF, as compared to that in control or other IPs. There is a possibility that patients with IPs might result in decreased clearance of inhaled dusts. However, the amounts of deposited particles including Si and Al in IPF without occupational exposure were also greater than those in other IPs. These results suggest that inorganic dust inhalation could induce directly or modulate indirectly the fibrosing process in IPF. In contrast, inorganic dust exposures did not appear to influence the pathogenesis of chronic HP and CVD.

Metal dust exposure is associated with an increased risk of the development of IPF[6,19,20] and other occupational factors including farming[19] or chronic exposure to sand or stone[19,20] have also been thought to be risk factors for IPF. The amounts of Si and Al detected in IPF are larger than those of other groups. It is noteworthy that our results are consistent with previous studies[7] during 1980's to 1990's, although we analyzed IPF diagnosed on the current criteria. The international consensus statement in 2000 defined the new methods of diagnosis, evaluation and management of patients with IPF. After the statement, we comprehend that interstitial pneumonias due to environmental dust exposures should be excluded from the diagnosis of IPF.[4] In this study, however, the amounts of Si and Al were detected more in lung tissues of IPF, even in IPF without occupational exposure. Therefore, unrecognized dust exposures are likely to be more frequent than we anticipate in IPF patients diagnosed on the current criteria. And, to the best of our knowledge, the present study is the first report to analyze the elemental deposition in lung tissues of chronic HP and IP with CVD.

Previous studies have described that alveolar macrophages of smokers contained more aluminium-silicate[21] and the elemental dust loads of Si and Al significantly increased in smokers according to the period of smoking history.[22] Our study showed no significant differences in age, gender and the quantitative index of smoking history among five groups. In addition, no significant differences were shown in the amounts of Si and Al deposition between smokers and non-smokers in IPF patients (data not shown).

Aluminium (Al) may be one of the elements which could induce occupational pulmonary diseases, and Al-induced pulmonary fibrosis has been described, showing fibrosis predominantly in the upper zone[3,23] and UIP pattern or less frequently DIP pattern.[2] Those cases were exposed to Al in their workplace,

and inhalation of aluminium oxides (Al_2O_3) is believed to be responsible for the disease. However, administration of Al_2O_3 resulted in minimal interstitial inflammation in the rat lung, whereas silica induced an increased collagen deposition.[24] Therefore, the role of Al as fibrogenic agent is not fully understood.

Particles of aluminium-silicate were found in all examined subjects including control lungs, which are consistent with the previous study.[8] Si and Al along with iron are known to be main components of soil, and principal constituents of igneous rocks are aluminium-silicate.[1] We hypothesize that most particles of aluminium-silicate were derived from soil distributed worldwide.

In conclusion, we demonstrated the possible involvement of inhaled inorganic-dusts as a responsible or a modulatory factor for IPF because unrecognized dust exposures are common even in IPF patients without occupational exposure. However, inorganic dust exposures did not appear to influence the pathogenesis of chronic HP and CVD. And it was suggested that elements which could be firmly associated with the disease process of IPF were Si and Al as components of aluminium-silicate. Some IPF patients without occupational exposure have the latent dust exposure and its pathogenesis might be partially similar to that of pneumoconiosis whch is an occupational lung disease. We propose that these cases should be excluded from IPF in the future study.

Acknowledgements

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