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<td>福原, 奈緒子</td>
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Cholesteryl-palmitate crystals in bronchoalveolar lavage fluid smears as a possible prognostic biomarker for choronic interstitial pneumonia: A preliminary study

(慢性間質性肺炎におけるBAL液スメアのパルミチン酸結晶の予後予測因子としての可能性）

福島県立医科大学大学院医学研究科
呼吸器内科学分野
呼吸器内科学講座

福原 奈緒子
Cholesteryl palmitate crystals in bronchoalveolar lavage fluid smears as a possible prognostic biomarker for chronic interstitial pneumonia: A preliminary study

Naoko Fukuhara\(^a\), Motoko Tachihara\(^a,\ast\), Yoshinori Tanino\(^a,\ast\), Junpei Saito\(^a\), Suguru Sato\(^a\), Takefumi Niihara\(^a\), Kenichi Misao\(^a\), Atsuro Fukuhara\(^a\), Xintao Wang\(^a\), Takashi Ishida\(^a\), Tetsuo Onami\(^a,\ast\), Mitsuru Munakata\(^a\)

\(^a\)Department of Pulmonary Medicine, School of Medicine, Fukushima Medical University, 1 Hirose-cho, Fukushima City, Fukushima 960-1295, Japan

Original article

ABSTRACT

Background: We observed cholesteryl-like crystals (Crystal X) in the bronchoalveolar lavage fluid (BALF) smears of patients with diffuse pulmonary disease. We analysed the clinical data of patients with and without crystals, and elucidated the structure of Crystal X and its concentration in the BALF.

Methods: Two hundred eighty-nine patients with diffuse pulmonary disease who underwent bronchoalveolar lavage (BAL) were analysed. The relationships between the presence and number of Crystal X in BALF smears and clinical parameters were investigated. Furthermore, structure determination and quantitative analyses of the crystals were performed.

Results: Seventy-five (26.1%) patients had Crystal X in their BALF. The crystals were frequently observed in patients with chronic interstitial pneumonia (CIP; 68/100 = 68.0%). Patients with Crystal X exhibited significantly higher serum Krebs von Loogern 6 antigen and surfactant protein-D levels (P < 0.01) and lower percentage vital capacity (P < 0.05) than patients without Crystal X. The number of crystals was significantly correlated with these parameters. The presence of crystals was also associated with a lower survival rate at 3 years after the BAL. The interfocal angles of the crystals were 126±2° and 144±2°, different from those of cholesteryl monohydrate crystals. Infrared absorption spectrometry showed Crystal X was cholesteryl palmitate. Its concentration was significantly higher in BALF with crystals than in BALF without crystals (P < 0.01).

Conclusions: Crystal X in the BALF of patients with diffuse pulmonary disease was identified as cholesteryl palmitate, which may be a useful prognostic biomarker for CIP.

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A piece of crystal in the BALF smear was obtained and its Fourier transform infrared spectrum was measured with transmitted light (Herschel FT/IR-660; JASCO Corp., Tokyo, Japan) using the JASCO Trion IRT-30 microscope unit (JASCO Corp.). The cumulative number was 100 and the resolution was 4 cm⁻¹. An MCT detector was used as the infrared detection element device. The IR Standard Database (Bio-Rad Sadler, Hercules, CA) was used to identify Crystal X.

For the quantitative analysis using HPLC, 3 mL of chloroform and chloroform benzene (i.e., the internal standard) were added to the BAL supernatant (0.3 mL). The chloroform layer was separated by shaking the mixture well for 5 min. The extracted solution was dried with anhydrous sodium sulfate, and the solvent was removed in vacuo. The residue was dissolved in hexane and analyzed by HPLC (Model LC-1500; JASCO Corp.). The analysis conditions were as follows: 15-cm column (Chiralcel OD-H; Daicel Corp., Tokyo, Japan); detector, ultraviolet (200 nm); eluent, methanol/isopropanol/water mixture (gradient, 96/0/4 for 0-4 min and 70/30/0 after 4 min); and flow rate, 1 mL/min at 25 °C.

2.6. Statistical analysis

The clinical parameters of the patients with and without Crystal X in the BALF smears were compared using the Student t test. The crystal (+) and crystal (−) subgroups were compared using the chi-square test. Spearman’s rank correlation analysis was used to determine the correlation between the clinical parameters and the number of Crystal X. The Kruskal–Wallis test was used to compare the number of Crystal X and the frequency of crystal-positive results between patients with IPF, those with other types of IIP, and those with IP-CTD. The Mann–Whitney test was used to compare the concentration of dissolved Crystal X in the BALF supernatant. All data were expressed as the mean ± standard error (SE), unless otherwise specified. A value of P ≤ 0.05 was accepted as significant.

3. Results

3.1. The presence of Crystal X in the BALF smears of patients with various forms of diffuse pulmonary disease

During the study period, 289 patients with diffuse pulmonary disease underwent BAL (Table 1). Crystal X in the BALF smears was observed in 45 of 130 patients with IIP, 15 of 40 patients with IP-CTD, four of 38 patients with sarcoidosis, four of seven patients with hypersensitivity pneumonia, three of 11 patients with organizing pneumonia, two of the three patients with alveolar proteinosis, one of the 11 patients with pneumocystis pneumonia, and one of three patients with radiation pneumonitis. There was no Crystal X in the BALF smear among patients with drug-induced pneumonia (n=10), eosinophilic pneumonia (n=10), pneumonia (n=7), or other diseases (n=19). The comparison between the CIP and non-CIP groups showed that the frequency of the presence of Crystal X was significantly higher in the CIP group (60/170 [35.3%] patients) than in the non-CIP group (15/119 [12.6%] patients); (P < 0.001). Only three patients had alveolar proteinosis; however, they had a high number of crystals in the BALF smear.

3.2. The association between the presence of Crystal X and the clinical parameters in patients with CIP

Among 170 patients with CIP, 60 patients (45 patients with IIP and 15 patients with IP-CTD) had Crystal X in the BALF
A piece of crystal in the BAF smear was observed and its Fourier transform infrared spectrum was measured with transmitted light (Herschel FTIR-660; JASCO Corp., Tokyo, Japan) using the JASCO lenses IR-T3 microscope unit (JASCO Corp.). The cumulative number was 100 and the resolution was 4 cm⁻¹. An AICT detector was used as the infrared detection element device. The IR Standard Database (Bio-Rad Sadtler, Hercules, CA) was used to identify Crystal X.

For the quantitative analysis using HPLC, 3 ml of chloroform and cholesterol benzoate (i.e., the internal standard) were added to the BAF smear (0.3 ml). The chloroform layer was separated by shaking the mixture well for 5 min. The extracted solution was dried with anhydrous sodium sulfate, and the solvent was removed in vacuo. The residue was dissolved in methanol (7 ml) and filtered. The filtrate was used for the HPLC analysis (Shimazu LC-15A; JASCO Corp.). The analysis conditions were as follows: 15-cm column (Chiralcel OD-H; Daicel Corp., Tokyo, Japan) detector, ultraviolet (200 nm); eluent, methanol/1-propanol; water mixture (gradient; 96/4 for 0-4 min and 70/30 after 4 min); and flow rate, 1 ml/min at 25 °C.

2.6. Statistical Analysis

The clinical parameters of the patients with and without Crystal X in the BAF smears were compared using the Student t test. The crystal (±) and crystal (−) subgroups were compared using the chi-square test. Spearman’s rank correlation analysis was used to determine the correlation between the clinical parameters and the number of Crystal X. The Kruskal-Wallis test was used to compare the number of Crystal X and the frequency of crystal-positive results between patients with IFP, those with other types of IF, and those with IP-CTD. The Mann-Whitney test was used to compare the concentration of dissolved Crystal X in the BAL supernatant. All data were expressed as the mean ± standard error (SE), unless otherwise specified. A value of P ≤ 0.05 was accepted as significant.

3. Results

3.1. The presence of Crystal X in the BAF smears of patients with various forms of diffuse pulmonary disease

During the study period, 298 patients with diffuse pulmonary disease underwent BAL (Table 1). Crystal X in the BAF smears was observed in 45 of 130 patients with IP, 15 of 40 patients with IP-CTD, four of 38 patients with sarcoidosis, seven of 24 patients with extrinsic allergic alveolitis, three of 11 patients with organizing pneumonia, two of the three patients with alveolar proteinosis, one of the 11 patients with pneumocystis pneumonia, and one of three patients with radiation pneumonitis. There was no Crystal X in the BAF smear among patients with drug-induced pneumonitis (n=10), eosinophilic pneumonia (n=10), pneumocystis pneumonia (n=7), or other diseases (n=19). The comparison between the CIP and non-CIP groups showed that the frequency of the presence of Crystal X was significantly higher in the CIP group (13/212 [6.2%] patients; P < 0.001). Only three patients had alveolar proteinosis; however, they had a high number of crystals in the BAF smear.

3.2. The association between the presence of Crystal X and the clinical parameters in patients with CIP

Among 170 patients with CIP, 60 patients (45 patients with IP and 15 patients with IP-CTD) had Crystal X in the BAL smear, while the remaining 110 patients (85 patients with IP and 25 patients with IP-CTD) did not. The clinical data were available from 148 patients. These data were compared between the crystal (±) subgroup (n=54) and the crystal (−) subgroup (n=94). The crystal (±) subgroup had significantly higher serum levels of Keras von Lungeren 6 antigen (KL-6) (1704 ± 187 U/ml vs. 1112 ± 27 U/ml in normal) and surfactant protein D (SP-D) (29.2 ± 24.8 ng/ml vs. 20.5 ± 17.9 ng/ml; P < 0.01 for both) and a significantly lower percentage vital capacity (%) VC value (74.9% ± 1.07% vs. 81.7% ± 2.16%; P = 0.015, Fig. 2) than the crystal (−) subgroup.

3.3. The association between the number of Crystal X in the BAF smears and the clinical parameters of patients with CIP

The correlation between the number of Crystal X in the BAL smears and the clinical parameters was analyzed. A significantly positive but weak correlation existed between the number of Crystal X and the serum levels of lactate dehydrogenase, KL-6, and SP-D. With regard to the parameters of the BAF, the total cell count and the number of neutrophils had a positive correlation with the number of Crystal X. A negative correlation existed between the number of Crystal X and the %VC value (Table 2). Patients with IFP (n=49), other types of IP (n=81), and IP-CTD (n=40) showed no difference in the number of Crystal X (5.0 ± 7.8, 8.1 ± 3.2, and 16.8 ± 6.4, respectively) and the frequency of crystal X (19/49 patients, 26/81 patients, and 15/40 patients, respectively). The outcomes were analyzed at 1 year after the BAL among the 121 patients for whom follow-up data were available. The survival rate was 76.3% (95/124 patients) in the crystal (±) subgroup and 89.3% (107/119 patients) in the crystal (−) subgroup, which indicated a significantly poorer outcome in the crystal (±) subgroup (P = 0.005).

4. Discussion

In the present study, we focused on Crystal X in the BAF smears of patients with diffuse pulmonary disease and obtained the following results. Crystal X was frequently present in patients with CIP. Among patients with CIP, those with Crystal X had significantly higher serum lactate dehydrogenase, KL-6, and SP-D levels, and the number of Crystal X in the BAF smears were correlated with the serum levels of these parameters. A year after the BAL, the survival rate was significantly lower in the patients with Crystal X. Infra-red absorption microspectroscopy and interferential angles measurement of Crystal X revealed that the crystals were actually cholesterol palmitate crystals. The concentration of cholesterol palmitate in the BAF was significantly higher in patients with CIP, compared to patients without them.

To the best of our knowledge, this is the first study to report the presence of cholesterol palmitate crystals in BAF smears. In 1968, Glancy et al. [8] reported 12 autopsy cases of pulmonary hypertension including the cases of diffuse interstitial lesions with granulomas that contained cholesterol-like crystals. They performed an X-ray crystallographic analysis of a granuloma from one patient, and revealed that the crystals were not cholesterol but was cholesterol palmitate, cholesterol stearate, or a mixture of the two. Villalba et al. [9] recently measured the serum lipid profiles in patients with pulmonary diseases by using liquid chromatography-tandem mass spectrometry. They reported that patients with IPF or familial interstitial
Fig. 3 – The infrared spectra of cholesterol-like crystals (Crystal X) and cholesteryl palmitate. The spectrum of Crystal X and cholesteryl palmitate is identical.

Fig. 4 – Quantitative analysis of cholesteryl palmitate in the bronchoalveolar lavage fluid (BALF) by high-performance liquid chromatography. The concentration of cholesteryl palmitate in the BALF is significantly higher in the cholesterol-like crystal-positive [crystal X (+)] group (n=6) than in the crystal X (−) group (n=4) (*P<0.05). The mean and standard error (SE) values are plotted.

Pneumonia (IP) had higher serum levels of cholesteryl palmitate than healthy individuals. Fireman et al. [10] reported two patients with IPF who had crystal structures in their BALF smears and concluded that they were cholesteryl crystals. However, the interfacial angles of the crystals represented in the figure in their article seemed to be different from the angles of cholesterol monohydrate crystals. Thus, we believe that the crystals may have been cholesteryl palmitate crystals, as in the present study.

Pulmonary surfactant is a complex that consists of lipids and proteins at a ratio of 9:1. Dipalmitoyl phosphatidylcholine, a phospholipid with two palmitic acids molecules, accounts for approximately 50% of the phospholipids in pulmonary surfactant [11] and it has a central role in surfactant activity [12]. Phosphatidylcholine has various acyl chains and the composition of these chains are altered in patients with diffuse pulmonary diseases and acute respiratory distress syndrome [13,14]. Fatty acids that constitute the pulmonary surfactant lipids are synthesized from carbohydrates and proteins via acyl-CoA. The synthesis ends at palmitic acid (C16). Stearic acid and oleic acid are thereafter endogenously synthesized through C16 fatty acid by elongation of very long chain fatty acid member 6 (Elov6). Sunaga et al. [15] recently reported that Elov6 expression was significantly decreased in the lungs of patients with IPF. They also reported an increase in the palmitic acid level in Elov6-deficient mice and its association with increased oxidative stress and pulmonary fibrosis. On the other hand, cholesterol esters such as cholesteryl palmitate have an important role in lipid transport in the body.

In recent years, the role of ATP-binding cassette transporter A (ABCA1) in lipid transport has been clarified. The molecule ABCA1 is involved in the elimination of cholesterol from the lung and its transport to the liver. In ABCA1-knockout mice, an increase in the amount of cholesterol in the lung and a considerable increase in the amount of cholesterol esters in the alveolar macrophages are observed with pathological findings similar to alveolar proteinosis [16]. Furthermore, ABCA3 has been shown to be involved in the transport of surfactant phospholipids [17]. Several studies have also reported an association between mutations of the ABCA3 gene and neonatal IP and adult IP [17–20].

The presence of cholesteryl palmitate crystals in the BALF smears in our study may be associated with an increase in the content of cholesteryl palmitate in the alveoli and may reflect abnormal lipid metabolism in patients with IPF, as described previously. The results of the present study, which include high serum levels of IP markers (e.g., KL-6 and SP-D), low %VC, and poor outcomes in patients with BALF
cholesterol palmitate crystals, lend support to the aforementioned notion.

5. Conclusions

This preliminary study revealed that cholesterol palmitate crystals are present in BALF sputum and may be a possible prognostic biomarker in patients with diffuse pulmonary disease. In addition, the presence of cholesterol palmitate crystals seemed to be associated with the pathophysiology of UIP. In the future, it would be worthwhile to conduct a prospective clinical study with quantitative analysis of cholesterol palmitate in BALF, and to promote basic research on the association between fatty acid esters such as cholesterol palmitate and pulmonary inflammation and fibrosis.

Conflict of interest

The authors have no conflicts of interest.

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