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Determination of desmosine in bronchoalveolar lavage fluids by time-resolved fluoroimmunoassay

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Abstract

Background: Urinary excretion of desmosine has been reported to be increased in patients with pulmonary fibrosis; however, several investigators have pointed out that measuring urinary desmosine is not a very useful indicator of lung wall destruction. We developed a sensitive time-resolved fluoroimmunoassay (TR-FIA) to identify trace amounts of desmosine in bronchoalveolar lavage fluid (BALF), and applied this method to analyze BALF samples from healthy subjects and patients with interstitial lung diseases.
Methods: In the proposed TR-FIA, a polystyrene strip was coated with desmosine-conjugated gelatin. The strip was then incubated with rabbit anti-desmosine antibody and the test solution. The desmosine bound to the solid phase and free desmosine in the sample or standard solution were allowed to compete to bind to the anti-desmosine. The solid-phase antibody was detected by Eu-complex conjugated anti-rabbit IgG.
Results: The detectable limit of desmosine was 50 fmol/ml in the TR-FIA developed in this study. TR-FIA showed low cross-reactivity against amino acids. BALF desmosine levels were significantly higher in patients with idiopathic pulmonary fibrosis and sarcoidosis compared with healthy subjects.
Conclusions: Desmosine levels in BALF may be useful to investigate lung disease.

Keywords: Bronchoalveolar lavage fluid; Desmosine; Time-resolved fluoroimmunoassay; Interstitial lung disease

1. Introduction

Elastin is an extracellular matrix protein that plays an important role in the stabilization of the structures of the lung, blood vessels and other organs that require flexibility and elasticity. Elastin is secreted into the intercellular space as a soluble precursor monomer, tropoelastin, and subsequently, the ε-amino groups of the peptidyl lysyl residues of tropoelastin are oxidatively deaminated by lysyl oxidase. The resulting aldehyde groups condense with other oxidized or unmodified lysyl residues to form crosslinks. The elastin contains characteristic crosslinks, including desmosine and isodesmosine, involving four lysyl residues [1–6]. The degradation of elastin-containing tissues that occurs in several widely prevalent diseases, such as pulmonary emphysema [7], adult respiratory distress syndrome [8], chronic obstructive pulmonary disease [9–11], cystic fibrosis [12] etc., has been reported to result in the urinary excretion of peptide-like fragments containing desmosine and/or isodesmosine; however, several reports have suggested that measuring urinary desmosine is not a very useful indicator of lung wall destruction [13–15].

Analyzing the components of bronchoalveolar lavage fluid (BALF), which is obtained by aspiration through a flexible fiberoptic bronchoscope after saline instillation [16], from patients with lung abnormalities may be of diagnostic and investigative value. Many reports have described determination methods of many components of BALF and the relation between these components and lung diseases [17–21]. There are also several reports about desmosine and isodesmosine in BALF; however, most concern experimental animals [15,22–26] with only a few studies on humans [27]. We determined desmosine in human BALF and to assess the relation between the desmosine level and interstitial lung diseases. Highly sensitive analytical

Abbreviations: TR-FIA, time-resolved fluoroimmunoassay; BALF, bronchoalveolar lavage fluid; IPF, idiopathic pulmonary fibrosis; PBS, phosphate-buffered saline.

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methods are required, because the concentrations of desmosine in BALF are extremely low. Several techniques have been used for the detection and determination of desmosine and isodesmosine in body fluids, such as HPLC [22,25,28,29], isotope dilution/HPLC [9,12,30], capillary electrophoresis [10,31,32], RIA [8,13–15,27,33–35], and EIA [11,23,24,36–38].

Time-resolved fluoroimmunoassay (TR-FIA) is an ultrasensitive immunoassay. Some lanthanide complexes are used for TR-FIA as labeling agents, which have fluorescence properties of a long fluorescence lifetime, large Stokes shift and sharp emission profile. Many applications of this technique have been reported [39–46]. The present study aimed to develop TR-FIA for determining desmosine, and to apply this method to the analysis of desmosine in BALF samples from 6 healthy subjects and 35 patients with two interstitial lung diseases, sarcoidosis and idiopathic pulmonary fibrosis (IPF).

2. Materials and methods

2.1. Reagents

The following chemicals were from the indicated sources: lyophilized desmosine (Elastin Products Company, Inc., Owensville MO); goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG (Organo Teknika N.V. Cappel Products, Westchester PA); diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic dianhydride (Dojin Chemical, Kumamoto, Japan); 3,3′,5,5′-tetramethylbenzidine (Nacalai Tesque Inc., Kyoto, Japan); soluble gelatin (Nippi Co. Ltd., Tokyo, Japan); gelatin (type A: from porcine skin, 300 bloom) (Sigma Chemical Co., St. Louis MO); and the enhancement solution for TR-FIA (Pharmacia Biotech, Uppsala, Sweden). All other chemicals were of analytical reagent grade. All solutions were prepared from water purified with a Milli-Q water system (Millipore Corp., Bedford MA). Polystyrene strips with 12 flat-bottomed wells were obtained from Flow Laboratories Inc. (McLean VA).

2.2. Buffers

Phosphate-buffered saline (PBS, pH 7.2) was used as a coating medium. When PBS was used for assay and washing buffers, soluble gelatin 10 g/l, and Tween® 20 5.0 g/l were added. Citrate buffer (100 mmol/l, pH 4.2) was used as the enzyme reaction medium.

2.3. Bronchoalveolar lavage and characteristics of subjects

Informed consent was obtained from all subjects and the study is approved by the institutional review board. A flexible bronchofiberscope with a tube was inserted into the bronchial space. Fifty milliliters of sterile saline was instilled and slowly aspirated through the tube. Six successive BALF samples were collected from each subject. BALF samples were filtered with a single layer of gauze to remove gross mucus, and centrifuged at 500 ×*g* for 10 min at 4 °C to remove cells. The supernatant was filtered through a membrane filter (pore size 0.45 μm), and stored at −20 °C. BALF samples were obtained from the following subjects: healthy subjects (6), and patients with IPF (11) and sarcoidosis (24). For this study, 246 BALF samples were obtained. All BALF samples were obtained by the same procedure at the same hospital. The characteristics of the study population are shown in Table 1. We diagnosed patients with sarcoidosis and IPF using normal chest X-ray, high-resolution chest CT, pulmonary function tests and surgical lung biopsy etc. The final diagnosis was confirmed by biopsy. The biopsy samples were reviewed by professional pathologists. The pathological diagnoses were as follows: sarcoidosis, demonstration of non-caseating granulomas, and IPF, demonstration of the usual interstitial pneumonia pattern. In addition, all patients with IPF satisfied the clinicoradiological criteria for IPF: exertional dyspnea, fine crackles on auscultation, clubbed finger, interstitial opacity on chest radiograph, restrictive pulmonary dysfunction with lowered diffusion capacity, and desaturation of blood oxygen during minimal exercise. On the other hand, these findings were not found in patients with sarcoidosis, except for lowered diffusion capacity.

Table 1
Characteristics of the study population

	Subject groups		
	Normal	Sarcoidosis	Idiopathic pulmonary fibrosis
Number of subjects	6	24	11
Age, y (mean±SD)	51.7±15.7	44.1±15.5	54.9±8.6
Age range, y	31–60	22–66	47–76
Sex, M/F	5/1	10/14	9/2
Current smokers [a]	2	4	7
Ex-smokers	0	2	3
Never smokers	4	18	1

[a] Stopped smoking more than 1 y previously.

2.4. Preparation of desmosine-conjugated gelatin

Desmosine-conjugated gelatin was prepared according to the method of Gunja-Smith [38]. Sodium periodate solution (0.1 mol/ml in 0.1 mol/l phosphate buffer, pH 4.4, 6.0 ml) was added to 20 mg of gelatin for 1 h in the dark, and the solution was dialyzed against 0.05 mol/l phosphate buffer (pH 7.5). Four milligrams of desmosine was added to the gelatin solution and 50 mg of sodium cyanoborohydride was added to the solution, which was left at room temperature for about 16 h. The reaction mixture was adjusted to pH 4.0, and then dialyzed against distilled water. The resulting solution was freeze-dried and stored at 4 °C.

2.5. Preparation of desmosine-conjugated BSA

Desmosine–BSA conjugate was prepared according to the previously reported method [38]. In brief, desmosine and BSA were conjugated using 1-ethyl-3-(3-dimethylaminopropyl)-3-carbodiimide in water containing pyridine. The conjugate was freeze-dried and stored at 4 °C.

2.6. Preparation of Eu-conjugated anti-rabbit IgG

Ten milligrams of diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic dianhydride was added to 1 ml of goat anti-rabbit IgG solution (10 mg/ml in 0.1 mol/l carbonate buffer, pH 9.5), and the mixture was stirred for 2 h at pH 9.5. The conjugate was separated from aggregated protein and the free chelating agent by size-exclusion chromatography (column, 2 cm × 36 cm Sephadex G-50; eluent, 0.01 mol/l carbonate buffer, pH 9.5). The conjugate fraction was lyophilized and dissolved in 1 ml of water. One hundred microliters of 0.02 mol/l $EuCl_3$ was added to the conjugate solution and the mixture was stirred for 2 h. The mixture was dialyzed against PBS to remove free Eu^{3+}, and stored at 4 °C.

2.7. Immunization

Equal volumes of desmosine–BSA conjugate solution (1 mg/ml in saline) and complete Freund's adjuvant were emulsified, and 1 ml of the emulsion was injected subcutaneously into rabbits at multiple sites. After 2 weeks, the rabbits received further immunization with 1 ml of emulsion consisting of 1 mg/ml desmosine–BSA conjugate and incomplete Freund's adjuvant (1:1). Immunization was continued every 2 weeks for 4 months. The animals were bled 10 days after the final immunization and the sera were stored in a freezer at −20 °C.

2.8. Pre-treatment of BALF samples

One hundred microliters of each BALF sample was evaporated to dryness in a small test tube under a vacuum. The tube was put into a vial connected to a Mininert® valve (20 ml). One hundred microliters of hydrochloric acid (6 mol/l) containing 0.01% phenol was added to the vial, which was sealed after degassing. The vial was then heated at 110 °C for 48 h to hydrolyze peptides. Following the removal of hydrochloric acid under a vacuum, the residue was dissolved in 1 ml of the assay buffer.