〈Original Article〉

Analysis of secretory immunoglobulin A concentration in saliva and exhaled breath condensate

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Summary Background: It is well known that secretory immunoglobulin A (SIgA) is present in saliva. However, the SIgA content in exhaled breath condensate (EBC) is unknown.

Objective: To determine the concentration of SIgA in EBC.

Methods: Eleven healthy male subjects donated saliva and EBC samples for SIgA analysis. The concentration of SIgA was measured using enzyme-linked immunosorbent assay (ELISA).

Results: The concentration of SIgA in saliva was 134.6 ± 63.2 μ g/mL with a range of 75.2-276.6 μ g/mL, and the concentration of SIgA in EBC was below the limit of detection for the ELISA.

Conclusion: Airway production of SIgA was unable to be measured in EBC specimens and, therefore, EBC specimens cannot be used to monitor SIgA concentration.

Key words: Saliva, Exhaled breath condensate, Secretory immunoglobulin A

1. Introduction

Salivary fluid is an exocrine secretion consisting of approximately 99% water, and contains a variety of electrolytes and proteins, including enzymes, immunoglobulins and other antimicrobial factors[1)]. These components interact and are responsible for the various functions attributed to saliva. Secretory immunoglobulin A (SIgA) is the largest immunological component of saliva with the ability to neutralize viruses, bacteria and toxins. It serves as an effective biological barrier by being able to aggregate bacteria, inhibiting their adherence to oral tissues[2, 3)].

The regulation of immune function is linked to the increased incidence of malignancy, infectious disease, and autoimmune diseases[4)]. Respiratory infections, such as pneumonia and influenza, including upper respiratory tract infections, are particularly common and can cause serious illnesses among the disabled, children[5, 6)], and the elderly[7)]. In preliminary studies, the production of SIgA in the mouth has been reported; however, production of SIgA in the airway and lungs is unknown.

The collection of exhaled breath condensate (EBC) is a recently developed technique for obtaining samples from the airways and lungs, which contains biomarkers including such as adenosine, hydrogen peroxide, isoprostanes, leukotrienes and cytokines[8)].

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However, to our knowledge, no studies examining SIgA in EBC have been reported. If SIgA is able to be detected in EBC, then the analysis of EBC could provide useful information about immune function in the airway and lungs of patients. Therefore, the purpose of this study was to determine whether SIgA was detectable in EBC.

2. Materials and methods

1. Subjects

The subjects included eleven healthy males (mean±SD; age: 25.2±2.6 years; height: 172.2±7.4 cm; weight: 64.1±7.1 kg). The procedures were explained to the subjects and informed written consent was obtained prior to sample collection. The investigation was conducted at the Hiroshima University after approval from the ethics committee of the Hiroshima University Graduate School of Health Sciences. All participation was voluntary and no payment for participation was made.

2. Preparations of saliva and EBC

After rinsing the mouth thoroughly with distilled water, timed whole saliva samples were collected by stimulating saliva production by chewing a sterile cotton swab (Salivette; Sarstedt, Germany) at a frequency of 60 chews per minute. Saliva was separated from the cotton swab by centrifugation at 1,400 × *g*. Then EBC was collected using a dedicated breath refrigeration circuit (ECoScreen®; Jaeger, Germany) according to European Respiratory Society/American Thoracic Society recommendations[8]. Subjects first rinsed their mouths with distilled water and breathed through a mouthpiece connected to a two-way non-rebreathing valve with a nose-clip on. Subjects were seated in a chair with no stimulation before the collection of EBC. After resting for 10 min, the condensate was collected for 15 min. The saliva and EBC samples were frozen at -30℃ and stored until used for analysis.

3. Assays

The concentration of SIgA was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial SIgA ELISA kit (K8870; Immundiagnostik AG, Germany). The concentrations of SIgA were assayed in duplicate by the following procedure and the mean calculated. Saliva samples were diluted 1:2000 with ELISA wash buffer (K8870WP; Immundiagnostik AG, Germany) and undiluted EBC were used for the assay. A pre-coated microtiter plate was washed five times with 250 μL ELISA wash buffer, and 100 μL calibrators (0, 22.2, 66.6, 200, 600 ng/mL), diluted saliva samples and undiluted EBC samples were added to individual wells. Samples were allowed to incubate for 1 h with shaking on a horizontal mixer at room temperature. Wells were washed and 100 μL peroxidase-labeled anti-SIgA antibody per well and incubated for 1 h. After washing again, the wells were incubated with 100 μL tetramethylbenzidine substrate solution for 10 min at room temperature. Finally, 50 μL per well stop solution was added and mixed briefly. The absorbance of each well at 450 nm was determined using an ELISA reader. The SIgA concentrations in saliva were corrected by the dilution factor of 2,000 for analysis. The SIgA values in EBC were used directly since they were not diluted.

A SIgA recovery study was undertaken by spiking EBC samples with 1 μg/mL SIgA to determine if EBC interferes with SIgA and the ability to recover SIgA from EBC samples. The recovery study was performed on other EBC samples collected from three healthy subjects, A, B and C. The concentration of SIgA was measured by ELISA using the following in-house method, as described previously[9-11]. A 96-well microtiter plate (Costar 3590; Corning, USA) was coated overnight at 4℃ with 2.4 μg/mL goat anti-human secretory component (bound and free; S1640; Sigma, USA) in carbonate-bicarbonate buffer (pH 9.6). After removing the coating solution, wells were blocked by the addition of 100 μL PBS containing 1% bovine serum albumin (A9418; Sigma, USA) for 2 h. Microtiter plates were washed three times with 100 μL phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST).

Spiked EBC samples were prepared by the addition of human SIgA (BA325; Acris Antibodies, Germany) to EBC samples to a final concentration of

1 μg/mL. Using human SIgA, 100 μL known concentrations of SIgA (0, 0.31, 0.63, 1.25, 2.5, 5, 10, 20 μg/mL) and spiked EBC samples were added to individual wells. Samples and standards were incubated for 2 h at room temperature. Wells were washed and 100 μL peroxidase-labeled anti-SIgA antibody (A0295; Sigma, USA) was added per well and incubated for 1 h. After washing again, the wells were incubated with 100 μL of peroxidase substrate solution (ABTS Tablet and ABTS Buffer; Roche, Germany) for 30 min at room temperature. Finally, 50 μL H_2SO_4 was added per well and mixed briefly. The absorbance of each well at 405 nm was determined using an ELISA plate reader (IMMUNO-MINI NJ-2003; InterMed, Japan). The lower limit of detection for this assay was determined to be 0.31 μg/mL. The percentage recovery from spiked EBC was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{\text{Measured concentration (}\mu\text{g/mL)}}{\text{Theoretical concentration (}\mu\text{g/mL)}} \times 100$$

3. Results and discussion

The SIgA concentration in saliva has been previously reported for healthy, young subjects[12, 13], the elderly[14, 15] and those with several diseases[6, 16-19]. However, the SIgA content in EBC is currently unknown. In the present study, the concentration of SIgA in saliva measured in young, healthy volunteers was determined to be 134.6±63.2 (mean± S.D.) μg/mL, with levels ranging from 75.2-276.6 μg/mL (Fig. 1). The saliva SIgA concentration detected in the present study was similar to that found in previous studies[12, 13]. Therefore, the ELISA used in the present study is adequate to measure the SIgA concentration in saliva.

To the contrary, the ELISA was unable to detect appreciable levels of SIgA in EBC samples collected from the same healthy subjects. The concentration of SIgA in EBC was below the limit of detection of 22.2 ng/mL for the commercial ELISA kit (Fig. 1). It has been previously reported that the mean total protein concentration in EBC, saliva and lung epithelial lining fluid were 4.6, 2,398 and 14,111 μg/mL, respectively, and the mean total protein concentration in saliva was approximately 500 times higher than for EBC[20]. As SIgA is one component of the total protein found in saliva and EBC (if present), the concentration of SIgA in EBC was predicted to be considerably lower than in saliva. In the present study, the result that SIgA in EBC was below the limit of detection for the assay and suggests that SIgA may still be present in EBC but could not be detect by ELISA. The ratio of the SIgA concentration in EBC to saliva

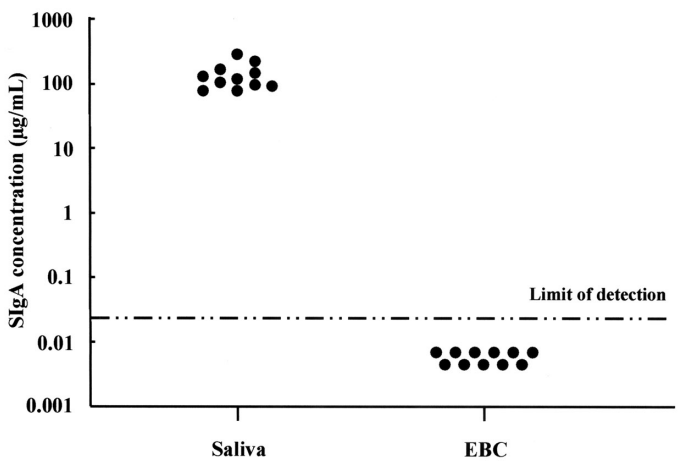

Fig. 1 Secretory immunoglobulin A (SIgA) concentrations in saliva and exhaled breath condensate (EBC) measured by ELISA. Dashed line represents the lower limit of detection for the assay.

Table 1 Recovery of secretory immunoglobulin A (SIgA) from spiked exhaled breath condensate specimens

Subjects	Theoretical concentration (μg/mL)	Measured concentration (μg/mL) [a]	SIgA Recovery
A	1.00	1.05	1.05%
B	1.00	1.15	1.15%
C	1.00	1.16	1.16%

[a] Values represent the mean of triplicate values.

could be estimated as <1:6000, and thus the absence of detectable SIgA in EBC samples was lower than expected.

To confirm these observations, we conducted a SIgA recovery study (Table 1). Recoveries of each spiked EBC were 105%, 115% and 116%, respectively. In the recovery study, the measured concentration was higher than the theoretical concentration. The ability to recover spiked SIgA from EBC samples suggested that there was no inhibitory effect caused by EBC for the measurement of SIgA concentration. In fact, there was a possible synergistic effect. Since the EBC samples were spiked with 1.00 μg/mL but the detected SIgA concentration was in fact higher, it was presumed that the estimated SIgA concentration range of the EBC sample was 0.05-0.16 μg/mL. However, this concentration range was extremely low and the concentration was lower than the limit of detection for the assay (0.31 μg/mL). Therefore, the synergistic effect observed in the SIgA recovery study casts doubts on the accuracy of the presumed concentration of SIgA in EBC.

In summary, our investigation shows that SIgA concentrations in EBC are considerably lower than those found in saliva samples. Thus, we conclude that airway production of SIgA was relatively low in EBC specimens; therefore, EBC specimens are unable to be used to monitor SIgA concentration in the airway.

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