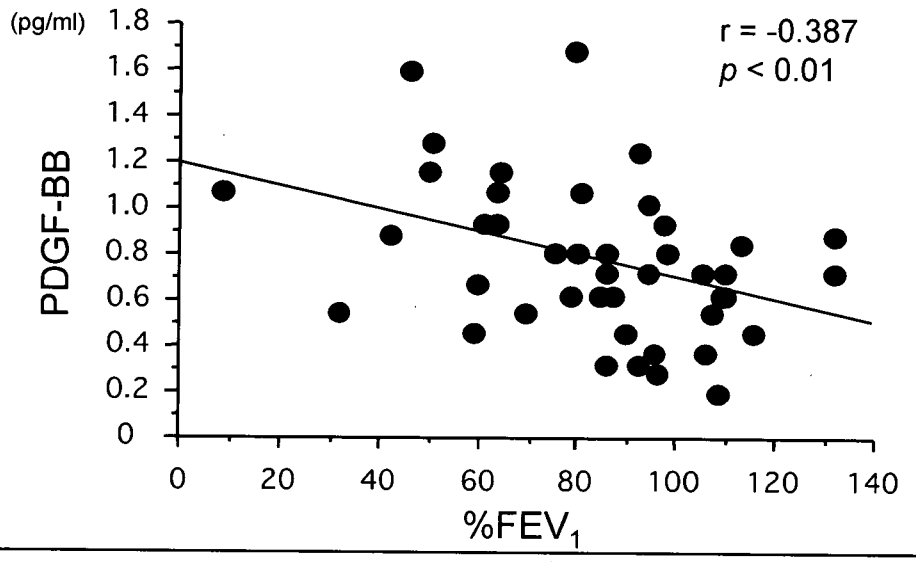
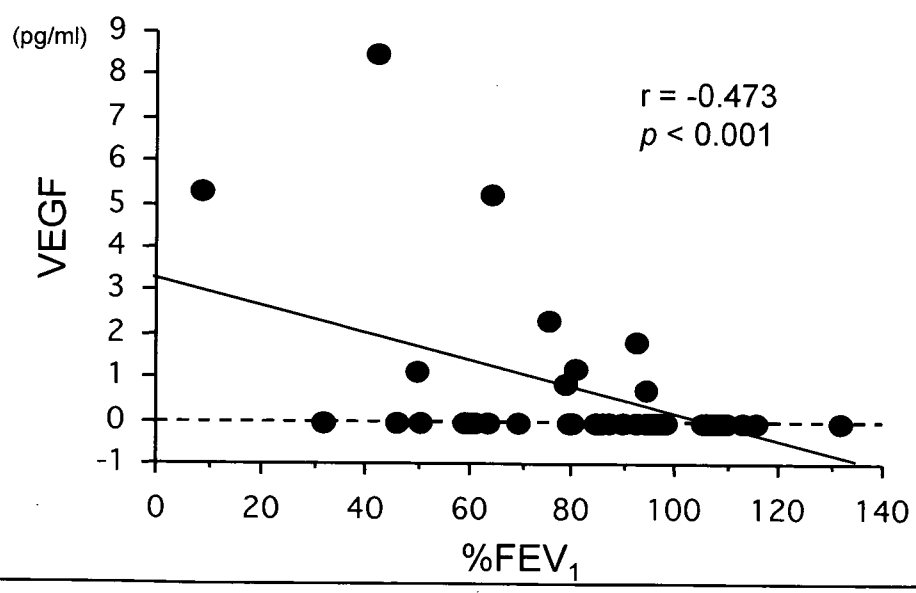
呼気凝縮液中のPDGF-BB濃度と閉塞性障害の相関
（大田）



呼気凝縮液中のVEGF濃度と閉塞性障害の相関
（大田）



Cytometric Beads Array System (CBA)

（一ノ瀬）

CBAの測定原理

- 蛍光強度が異なるビーズに、標的物質のcapture抗体がcoatingされている。
- サンプルおよびdetection用抗体を反応させる。
- Flow cytometryで、ビーズの蛍光強度により抗原を検出する
- 抗体の蛍光量により抗原量を同時測定する。

CBAの特徴

- 少量のサンプル (50 μL) で多種類の物質を、同時に測定することができる。
- 測定に要する時間は約4時間と、従来のELISA法の半分程度である。
- 感度は従来のELISA法とほぼ同等である。
- 専用のソフトウェアで自動解析が可能である。

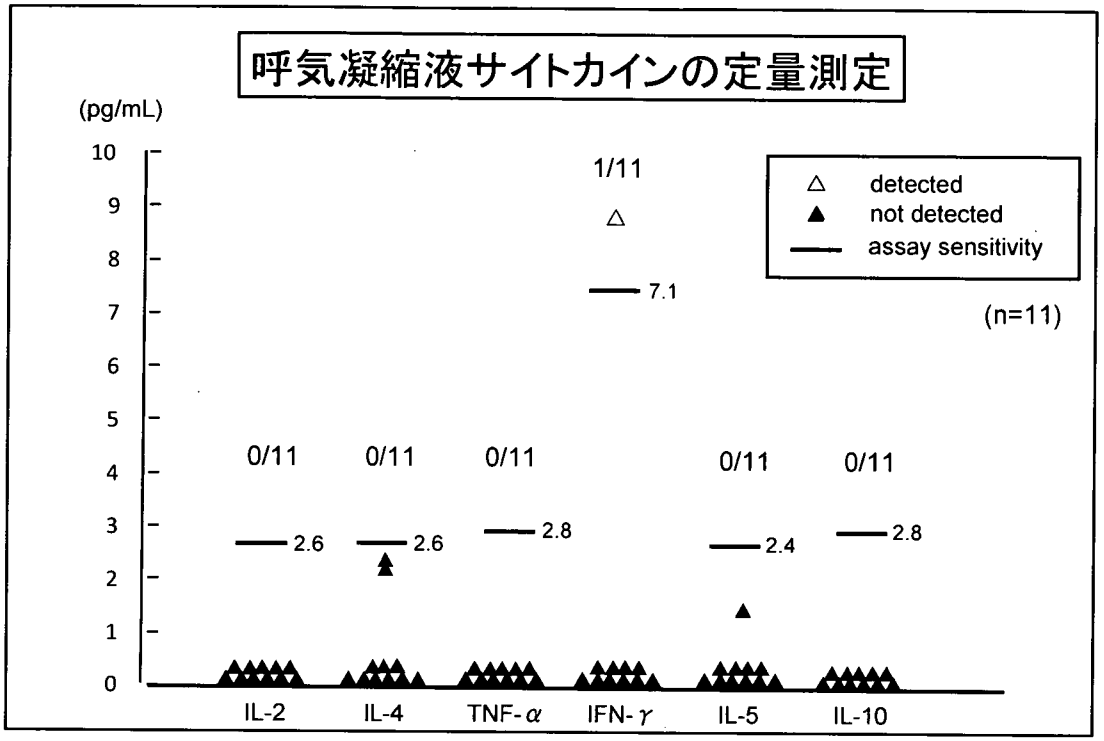
呼気凝縮液サイトカイン・ケモカイン発現と呼吸機能

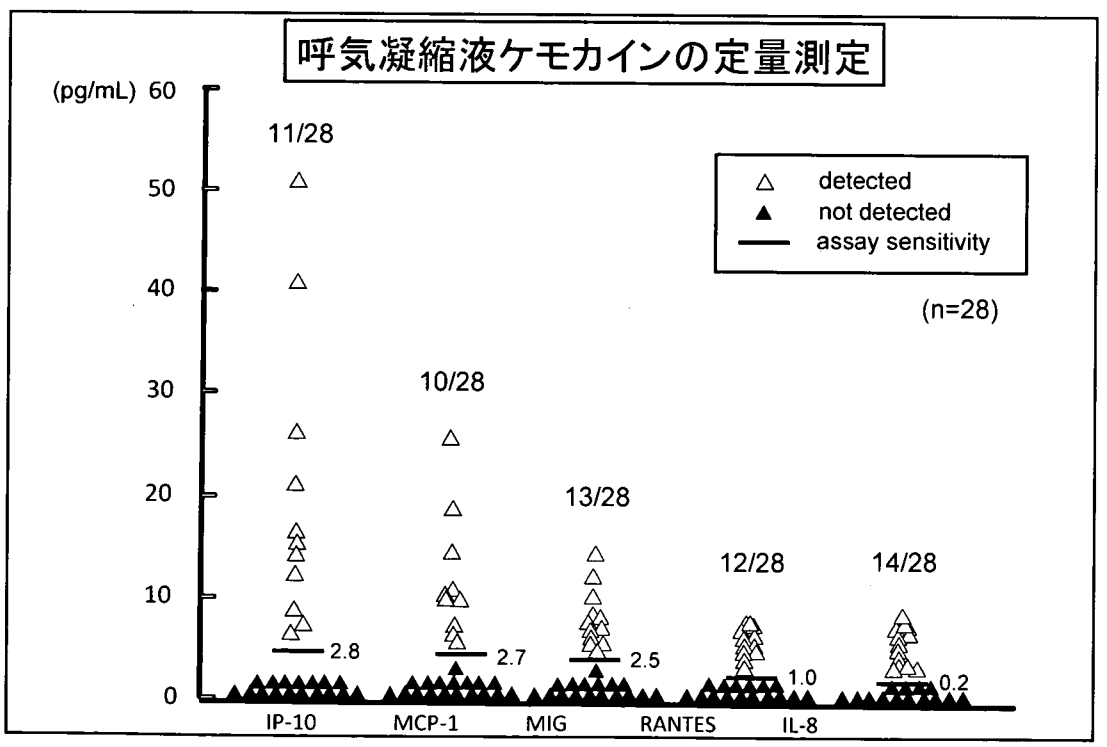
（一ノ瀬）

Subject demographics

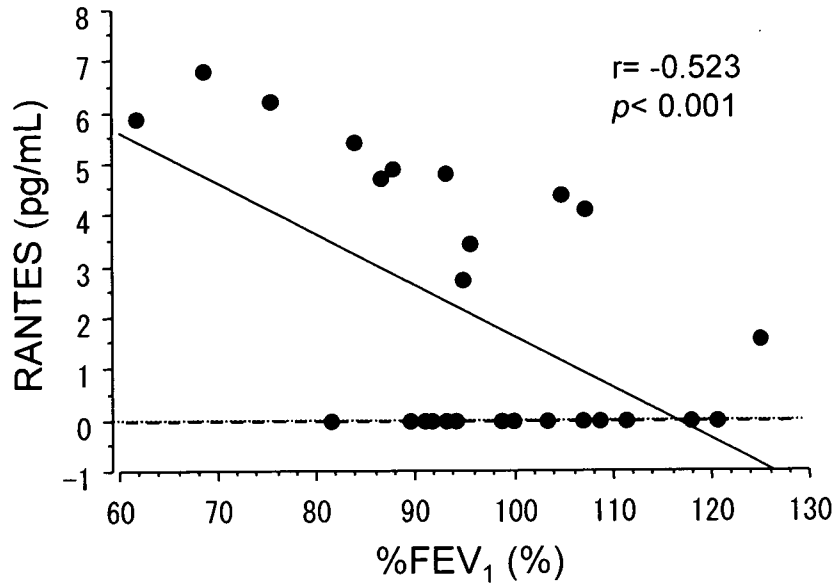
Number	24
Age (yrs)	51.2±3.1
Sex (F / M)	17 / 7
FVC (L)	3.23±0.16
FEV_1 (L)	2.51±0.13
FEV_1% (%)	77.6±1.5
%FEV_1 % (%)	98.8±2.8

mean±SE
FVC: forced vital capacity
FEV_1: forced expiratory volume in one second


呼気凝縮液サイトカインの定量測定
(pg/mL)
10
9
8
7
6
5
4
3
2
1
0
1/11
7.1
detected
not detected
assay sensitivity
(n=11)
0/11
0/11
0/11
0/11
0/11
2.6
2.6
2.8
2.4
2.8
IL-2
IL-4
TNF-α
IFN-γ
IL-5
IL-10


呼気凝縮液ケモカインの定量測定
(pg/mL) 60
50
40
30
20
10
0
11/28
10/28
13/28
12/28
14/28
detected
not detected
assay sensitivity
(n=28)
2.8
2.7
2.5
1.0
0.2
IP-10
MCP-1
MIG
RANTES
IL-8

呼気凝縮液中のRANTES濃度と閉塞性障害の相関



呼気凝縮液検査の臨床応用化
2）評価分子の定量化・病態との関連性

- Empore+HPLC, Luminex System, Cytometric Beads Array System で、LTB_4, IP-10, MCP-1, MIG, IL-8, RANTES, IGF-1, FGF, PDGF-BB, VEGFの11種の分子が50-100%の患者で定量測定できた。

- LTB_4濃度は、軽症に比べ重症喘息で有意に高値で、喀痰の好中球成分が多いほど高値であった。
  →難治性喘息の検出・管理への応用

- RANTES, PDGF-BB, VEGF濃度は閉塞性障害と有意な負の相関を認めた。
  →喘息の管理指標への応用

3年間の研究成果のまとめ

1. 妥当性
- 呼気凝縮液検査は安全かつ妥当な気道炎症評価法である

2. 有用性
- 炎症性メディエータ・サイトカイン・ケモカインの喘息気道での上昇が把握可能で、気道閉塞・過敏性といった喘息病態とも相関性を持つ

3. 臨床応用（病態との関連性・評価分子の定量化）
- ステロイド感受性分子と抵抗分子
  →治療方針の選択に有用な可能性
- 11種の評価分子 (Cys LTs, LTB_4, IP-10, RANTES, MCP-1, MIG, IL-8, FGF, IGF-1, PDGF, VEGF)が定量測定でき、 評価分子と喘息病態に関連性を認めた：Cys LTs (喘息発作の予測因子)、LTB_4 (好中球浸潤)、RANTES, PDGF, VEGF (閉塞性障害)
  →治療効果判定や難治群の予測への応用

喘息管理における呼気凝縮液検査 今後の課題・応用

- 他の炎症モニタリング法（呼気ガス分析）との組み合わせによって、より臨床上の有用性を高める
- 炎症物資の同定による個別化治療と従来型治療との費用対効果に関する検討
- 高齢者喘息のCOPDとの鑑別診断における有用性の検討

Ⅴ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ichikawa T, Ichinose M, et al:	Possible impact of salivary contamination on cytokine analysis in exhaled breath condensate	Analytical Chemistry Insights	2	85-92	2007
Ueshima K, Ichinose M, et al:	The Influence of Free 3-Nitrotyrosine and Saliva on the Quantitative Analysis of Protein-Bound 3-Nitrotyrosine in Sputum	Analytical Chemistry Insights	1	1-7	2007
Yamagata T, Ichinose M, et al:	Overexpression of CD-11b and CXCR1 on Circulating Neutrophils: Its Possible Role in COPD	Chest	132	890-899	2007
Tomaki M, Ichinose M, et al:	Decreased expression of antioxidant enzymes and increased expression of chemokines in COPD lung	Pulm Pharm Ther	20	596-605	2007
Akamatsu K, Ichinose M, et al:	Improvement of pulmonary function and dyspnea by tiotropium in COPD patients using a transdermal β_2-agonist	Pulm Pharm Ther	20	701-707	2007
市川朋宏, 一ノ瀬正和	呼気による気道炎症評価法	呼吸器疾患	5	134-136	2007
一ノ瀬正和, 田村 弦	あらたな喘息診断法の可能性	医学のあゆみ	220	958-961	2007
一ノ瀬正和	COPD と喘息の鑑別診断	クリニカル プラクティス	26	495-498	2007
市川朋宏, 一ノ瀬正和	誘発喀痰	綜合臨床	56	1876-1881	2007
一ノ瀬正和	分子生物学時代のオランダ仮説	THE LUNG perspectives	15	25-30	2007
杉浦久敏, 一ノ瀬正和	COPD と喘息との比較 COPD と喘息はどう違うのか？	Modern Physician	27	1463-1468	2007
一ノ瀬正和	薬物療法の新展開-生理的意義と新しい可能性-	日本臨床	65	689-695	2007
杉浦久敏, 一ノ瀬正和	気道炎症と気道リモデリング	喘息	20	27-32	2007

杉浦久敏, 一ノ瀬正和	COPD－炎症性メディエーターの測定と気道炎症の評価－	日胸	66	917-923	2007
市川朋宏, 一ノ瀬正和	気管支喘息と COPD-病態の類似点と相違点-	日胸	66	113-120	2007

VI. 研究成果の刊行物

Possible Impact of Salivary Influence on Cytokine Analysis in Exhaled Breath Condensate

T. Ichikawa, K. Matsunaga, Y. Minakata, S. Yanagisawa, K. Ueshima, K. Akamatsu, T. Hirano, M. Nakanishi, H. Sugiura, T. Yamagata and M. Ichinose

The Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, Wakayama, Japan.

Abstract

Background: Exhaled breath condensate (EBC) is thought to contain substances of the lower airway epithelial lining fluid (ELF) aerosolized by turbulent flow. However, contamination by saliva may affect the EBC when collected orally.

Objective: The purpose of this study was to compare the cytokine expression levels in EBC with those in saliva, and to clarify the influence of saliva on cytokine measurements of EBC.

Methods: EBC and saliva samples were obtained from 10 adult subjects with stable asthma. To estimate differences in the contents of substances between EBC and saliva, the total protein concentration of each sample was measured. Further, we also measured the total protein concentration of ELF obtained from another patient group with suspected lung cancer using a micro sampling probe during bronchoscopic examination and roughly estimated the dilution of EBC by comparing the total protein concentration of EBC and ELF from those two patient groups. The cytokine expression levels of EBC and saliva from asthmatic group were assessed by a cytokine protein array.

Results: The mean total protein concentrations in EBC, saliva and ELF were 4.6 μg/ml, 2,398 μg/ml and 14,111 μg/ml, respectively. The dilution of EBC could be estimated as 1:3000. Forty cytokines were analyzed by a cytokine protein array and each cytokine expression level of EBC was found to be different from that of saliva. Corrected by the total protein concentration, all cytokine expression levels of EBC were significantly higher than those of saliva.

Conclusion: These results suggest that the salivary influence on the cytokine assessment in EBC may be negligible.

Keywords: asthma, cytokine, chemokine, growth factor, epithelial lining fluid

Introduction

Exhaled breath condensate (EBC) is collected by cooling exhaled air. Collecting EBC is a noninvasive and repeatable method, and many researchers have reported the usefulness of EBC for measuring airway inflammatory molecules in respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Kharitonov and Barnes, 2004; Liu, J and Thomas, 2005; Barnes et al. 2006). EBC potentially reflects the volatile and non-volatile substances derived from all the airways. Non-volatile substances in airway epithelial lining fluid (ELF) are thought to be aerosolized by the turbulent flow generated mainly in the lower airway. Then, they are diluted by exhaled water vapor and incorporated into EBC. The dilution of EBC has been estimated using the conductivity, total cations and urea of plasma and EBC (Effros et al. 2004). When EBC is collected orally, it could contain substances derived from saliva (Silkoff et al. 2006). Some solutes measured in EBC such as amylase, and eicosanoids have been examined as markers of salivary contamination (Kharitonov and Barnes, 2001; Griese et al. 2002). The EBC pH from COPD patients has been reported to be unsuitable as a marker of airway acidification because it is affected by the volatile salivary acid (Effros et al. 2006). Leukotrien B4 in EBC has been reported to be derived from saliva (Gaber et al. 2006). However, the proportional contribution of saliva on the cytokine assessment of EBC has not yet been sufficiently studied.

Several molecules such as eicosanoids, chemokines and cytokines have been measured in EBC with the use of various assays such as a specific enzyme immunoassay (Shahid et al. 2002; Ko et al. 2006), gas

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chromatography/mass spectrometry (Cap et al. 2004), and cytometry beads array (Sack, U et al. 2006, Gessner et al. 2005). However, the precise molecular properties in EBC have not been clearly determined because the concentration of molecules measured in EBC is extremely low and there is relatively high variability in repeated measurements (Horvath et al. 2005; Hunt, 2002). We previously reported that a cytokine array assay was useful for cytokine analysis of EBC obtained from asthmatic patients (Matsunaga et al. 2006). Although this was a semiquantitative assay, the expression levels of forty cytokines could be simultaneously measured and we found that several cytokines, such as interleukin (IL)-4, IL-8, IL-17, tumor necrosis factor-alpha (TNF-α), regulated upon activation, normal T-cell expressed (RANTES), interferon (IFN)-γ-inducible protein 10 (IP-10), transforming growth factor-beta 1 (TGF-β1), macrophage inflammatory protein 1 alpha (MIP-1α) and MIP-1β were more up-regulated in EBC from asthmatic patients than in that from healthy controls (Matsunaga et al. 2006). However, the concern about the salivary influence on the results remains.

In the present study, we compared the cytokine expression levels in EBC with those in saliva to evaluate the influence of saliva on the cytokine measurements of EBC. Furthermore, we estimated the extent to which the contents in ELF and saliva contribute to EBC by comparing the total protein concentration of EBC and saliva from an asthmatic group with that of ELF from another patient group who had undergone bronchoscopic examination.

Materials and Methods

Study subjects

For EBC and saliva collection, adult patients with stable asthma were recruited. All patients met the American Thoracic Society criteria for asthma (The official statement of the American Thoracic Society, 1986). Stable asthma was defined as an absence of unscheduled physician visits for asthma care, unchanged use of asthma medication for maintenance therapy, and stable use of rescue medication for at least 4 weeks before sample collection. The patients who used regular anti-asthmatic medications except inhaled corticosteroids, had oral diseases or were current smokers were excluded. Patients planning to have a bronchoscopic examination were recruited for ELF collection. Patients with suspected infectious disease or high risk of bleeding were excluded. This study was approved by the ethics committee of Wakayama Medical University and all patients gave written informed consent.

Ten patients were recruited for each subject group. Among the asthmatic patients, seven required daily therapy with inhaled corticosteroids equivalent to a dose of 400 μg or more fluticasone. Two patients were ex-smokers. Two patients had a hypertension and used antihypertensive drugs. Among the patients for ELF collection, four were current smokers and four patients were ex-smokers. Two patients had stage II COPD without medication. Two patients had hypertension and one patient used antihypertensive drugs. After bronchoscopic examinations, seven patients were diagnosed as having lung cancer (three patients as adenocarcinoma, three patients as non-small cell lung carcinoma, one patient as metastatic lung carcinoma) and three patients exhibited no abnormal findings. The characteristics of the study subjects are listed in Table 1.

Study design

This study was cross-sectional. Subjects with asthma attended the outpatient clinic at the Wakayama Medical University Hospital on one occasion for clinical examination, spirometry, and collection of both EBC and saliva samples. Subjects planning to have a bronchoscopic examination were admitted to the hospital and underwent spirometry and bronchoscopic examination. Spirometry was performed the day before the broncoscopic examination.

EBC, saliva and ELF collection

The EBC was collected by using a condenser, which permitted noninvasive collection of con-

Table 1. Characteristics of study subjects.

	Asthmatics	ELF group	p value
Subject number	10	10	
Age(yr)	44.6 ± 5.4	63.1 ± 5.3	0.025
Sex female/male	5/5	1/9	
FVC(L)	3.35 ± 0.16	3.51 ± 0.29	0.824
FEV_1 (L)	2.48 ± 0.18	2.55 ± 0.25	0.647
FEV_1%(%)	73.9 ± 3.6	74.2 ± 5.1	0.960
%FEV_1(%)	84.5 ± 6.5	87.9 ± 5.2	0.691

FEV_1: forced expiratory volume in one second; FVC: forced vital capacity values are means ±SE.

densed exhaled air by freezing it to –20 °C (Ecoscreen; Jaeger, Hoechberg, Germany) (Montuschi et al. 2000). The subjects breathed through a mouthpiece and a two-way non-rebreathing valve, which also served as a saliva trap. Subjects were asked to breath at a normal frequency and tidal volume, wearing a nose-clip, for 20–30 minutes. The collected EBC was melted and transferred to 1 ml Eppendorf tubes and immediately stored at –80 °C until investigation. The mean volume collected was approximately 2–3 ml.

Saliva was collected by expectoration into plastic tubes following EBC collection. Collected saliva samples were centrifuged at 300 g for 5 minutes and the separated supernatant was stored at –80 °C until investigation.

ELF samples were collected using bronchoscopy with microsampling probes (model BC-402C; Olympus, Tokyo, Japan) as previously described (Yamazaki et al. 2003). Briefly, the probe comprised a 2.5 mm outer diameter polyethylene sheath and an inner 1.9 mm-polyester fiber rod probe attached to a stainless steel guide wire. This probe immediately absorbs fluid. A flexible fiberoptic bronchoscope (model BF-P240; Olympus) was inserted into the right or left main bronchus after local anesthesia of the upper respiratory tract was achieved with a few milliliters of 2–4% lidocaine. After the channel of the bronchofiberscope was flushed with air, the microsampling probe was inserted through the channel into a main bronchus. Then, the inner probe was advanced slowly into the airway, and sampling of ELF was performed by placing the probe gently at a site of the targeted bronchial wall for 10 seconds. To avoid blood contamination, the subject was asked to hold a breath while the inner probe was placed at the bronchus. The inner probe was withdrawn into the outer tube, and both devices were withdrawn simultaneously. If visible blood contamination on the inner probe was detected, we inserted a microsampling probe again and recollected the ELF sample. The wet inner probe was cut 2cm from its tip. Three collected tips were placed in a tube and centrifuged at 10,000g for 10min. The solution was transferred to a new tube and stored at –80 °C until investigation. The probe was the dried and weighed again to measure the ELF volume recovered.

Cytokine assessment and detection of the protein concentration were performed within 4 weeks after collection of the EBC, saliva and ELF samples.

Cytokine measurements

Human Inflammation Antibody III (Ray Biotech Inc., Norcross, Ga, U.S.A.), consisting of 40 different cytokine and chemokine antibodies spotted in duplicate onto a membrane, was utilized (Huang, 2001a; Huang, 2001b; Lin et al. 2003; Turtinen et al. 2004). Briefly, the membranes were blocked with 10% bovine serum albumin in Tris-buffered saline, and then 1.0 ml each of EBC and saliva obtained from the asthmatic subjects was added to each membrane and incubated at room temperature for 2 hours. The membranes were washed, and 1.0 ml of primary biotin-conjugated antibody was added and incubated at room temperature for 2 hours. Following a thorough wash, the membranes were incubated with 2.0 ml of horseradish peroxidase (HRP)-conjugated streptavidin at room temperature for 1 hour. The intensity of signals was detected directly from the membranes using a chemiluminescene imaging system (Luminocapture AE6955; Atto Co., Tokyo, Japan). Exposure times ranged from 30 seconds to 2 minutes. Chemiluminescence was quantified with Atto imaging and analysis software. HRP-conjugated antibody served as a positive control at six spots and was also used to identify the membrane orientation. For each spot, the net intensity gray level was determined by substracting the background gray levels from the total raw intensity gray levels. The relative intensity levels of the cytokines were normalized with reference to the amounts of cytokines present on the positive control in each membrane on the following basis: average of the cytokine spot intensity levels/ average of the positive control spot intensity levels, indicated as a percentage. Both the cytokine detection level and the within-subjects reproducibility of this array have been described in a previous report (Matsunaga et al. 2006).

Total protein measurements in EBC, saliva, and ELF

The total protein concentrations of the saliva and ELF samples were measured with a Bradford protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) (Bradford 1976). The protein concentration of EBC was measured with a Micro BCA Protein Assay kit (Pierce, Rockford, IL, U.S.A.) (Smith et al. 1985).

Pulmonary function

Forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were measured

with a Vitalograph Pneumotrac 6800TM (Vitarograph Co., Ennis, Ireland) according to a standard procedure (Standardization of Spirometry, 1994).

Statistical analysis

Statistical analysis was performed using the statistical software package Stat View (Abacus Concepts, Berkley, CA). Comparisons of the characteristics between two groups (asthmatic groups and ELF group) were performed by the Student's t-test. Comparisons of the total protein concentration between EBC and saliva were performed by the Paired t-test. Comparisons of cytokine expression levels between EBC and saliva were performed by the Wilcoxon signed-ranks test. The results were reported as means ± SE except cytokine expression levels. Each cytokine expression level was expressed as median (interquartile range (IQR)). Significance was defined as a p value of less than 0.05.

Results

Total protein concentration

The total protein concentration was detectable in all EBC, saliva, and ELF samples. The mean total protein concentrations of EBC, saliva and ELF were 4.6 ± 1.1 μg/ml, 2,398 ± 379 μg/ml and 14,111 ± 3,477 μg/ml, respectively (Table 2). The ratio of the total protein concentration between EBC, saliva, and ELF was approximately 1:500:3000.

Comparison of the cytokine expression levels between EBC and saliva

The expression levels of each cytokine in EBC and saliva are shown in Table 3. The cytokines were lined up in the order of their expression levels in EBC with the highest level first. The cytokine order of saliva was lined up to match that of EBC. Eotaxin, eotaxin-2, granulocyte-colony stimulating factor (G -CSF) and granulocyte-macrophage colony stimulating factor (GM -CSF) showed significantly higher expression levels in EBC than in saliva (eotaxin: $p = 0.013$, eotaxin-2: $p = 0.037$, G-CSF, GM-CSF: $p = 0.028$). In contrast, the expression levels of IL-8, tissue inhibitor of metalloproteinases-2 (TIMP2), IL-1β, monocyte chemoattractant protein 1 (MCP-1), soluble TNF receptor I (sTNF-RI), intercellular adhesion molecule 1 (ICAM-1) and IL-7 were significantly higher in saliva (IL-8, TIMP2, MCP-1: $p = 0.005$, sTNF-RI, ICAM-1: $p = 0.009$, IL-1β: $p = 0.017$, IL-7: $p = 0.047$). The differences in the other cytokines between saliva and EBC were not significant.

Table 2. Total protein concetration of each sample.

	EBC	Saliva	ELF
total protein concentration (μg/ml)	4.6 ± 1.1	2,398 ± 379*	14,111 ± 3,477**†

The samples of both EBC and saliva were obtained from the same asthmatic patients. ELF samples were obtained from another subject group who had undergone bronchoscopic examination. The ratio of the total protein concentration between EBC, saliva, and ELF was approximately 1:500:3000. *$P = 0.0001$ compared with the value of EBC **$P = 0.0007$ compared with the value of EBC †$P = 0.0036$ compared with the value of saliva. Values are means ± SE.

Comparison of the cytokine expression levels between EBC and saliva corrected by the total protein contents

After correction by the total protein contents, the expression levels of all cytokine in EBC were significantly higher than those in saliva. Especially, seven cytokines (IL-8, TIMP-2, sTNF-RI, MCP-1, IL-1β, ICAM-1 and IL-7), which were more up-regulated in saliva than in EBC before protein correction, also showed significantly higher expression levels in EBC than in saliva (Figs. 1A and 1B). Likewise, the corrected expression levels of the eight cytokines (IL-4, IL-17, TNF-α, RANTES, IP-10, TGF-β1, MIP-1α and MIP-1β), which had been reported to be more up-regulated in EBC from asthmatics (Matsunaga K et al. 2006), were significantly higher in EBC than in saliva (Figs. 1C and 1D).

Discussion

In the present study, EBC and saliva were found to have different levels cytokine expression. Among the examined molecules, eotaxin, eotaxin-2, GM-CSF and G-CSF were significantly more up-regulated in EBC than in saliva, although the total protein concentration of saliva was 500 times higher than that of EBC. Corrected by the total protein contents of the EBC and saliva, all cytokine expression levels measured in this study were significantly higher in EBC than in saliva. These data suggest that the effect of salivary contamination on the cytokine assessment in EBC would be

Table 3. Each cytokine expression level in ebc and saliva.

Cytokines	EBC(%)	Saliva(%)	p value	Cytokines	EBC(%)	Saliva(%)	p value
TNF-β	41.4 (36.6–51.6)	43.8 (33.8–52.6)	0.721	IL-16	10.3 (8.5–21.9)	11.4 (3.5–17.2)	0.386
IP-10	37.0 (28.0–37.9)	36.6 (28.2–50.1)	0.575	M-CSF	10.3 (8.1–14.2)	12.4 (8.9–21.8)	0.139
TNF-α	34.3 (25.9–43.8)	32.9 (24.8–46.3)	0.333	GCSF	9.9 (5.2–12.1)	5.6 (2.3–6.9)	0.028
RANTES	25.8 (21.4–44.5)	29.7 (15.9–49.2)	0.575	IL-2	9.9 (8.0–26.9)	10.9 (4.4–22.1)	0.799
TGF-β1	21.8 (16.3–31.5)	24.0 (14.3–36.8)	0.959	MIP-1α	9.6 (8.2–16.8)	18.1 (7.1–19.5)	0.575
MIP-1β	20.8 (16.8–30.3)	32.9 (19.6–52.4)	0.241	MCP-1	8.7 (6.8–15.1)	51.2 (38.7–62.5)	0.005
IL-15	15.9 (6.2–26.6)	11.5 (5.4–14.3)	0.114	IL-17	8.2 (5.1–10.5)	6.1 (5.4–7.1)	0.386
EOTAXIN2	15.5 (14.5–23.0)	12.4 (5.4–20.3)	0.037	IL-1β	7.9 (4.6–20.9)	56.3 (9.3–199.6)	0.017
IL-8	15.5 (12.2–19.7)	174.8 (108.1–358.8)	0.005	IL-6	6.9 (4.4–9.4)	5.9 (2.4–7.9)	0.386
PDGF-BB	14.0 (10.6–17.5)	16.8 (11.3–23.2)	0.386	MIG	6.7 (5.0–6.9)	8.7 (4.4–13.1)	0.333
IL-6sR	13.3 (7.6–14.6)	11.9 (9.2–15.9)	0.799	I-309	6.5 (3.0–8.0)	4.6 (1.3–7.3)	0.241
EOTAXIN	13.2 (10.5–21.8)	10.1 (5.4–17.8)	0.013	IL-12p40	4.8 (3.4–7.2)	8.1 (2.9–12.3)	0.799
sTNF R II	13.1 (10.5–14.1)	16.2 (13.5–19.0)	0.386	IFN-γ	4.7 (4.2–11.3)	5.1 (1.4–6.7)	0.093
TIMP-2	12.9 (9.0–15.7)	56.7 (48.2–110.4)	0.005	GM-CSF	4.6 (3.2–5.6)	2.3 (1.8–4.8)	0.028
IL-10	12.5 (10.0–20.6)	18.6 (13.1–25.0)	0.093	IL-13	4.6 (1.6–13.0)	5.4 (2.4–14.3)	0.508
MIP-1δ	12.5 (5.9–18.7)	19.3 (5.2–32.4)	0.169	MCP-2	4.1 (3.1–8.4)	5.0 (4.6–9.1)	0.508
IL-3	11.5 (7.0–21.5)	5.8 (3.6–13.8)	0.114	ICAM-1	4.0 (2.9–6.1)	14.9 (13.4–19.0)	0.009
sTNF-R I	11.1 (10.6–19.3)	32.6 (20.6–45.9)	0.009	IL-7	3.3 (2.3–5.4)	5.4 (4.3–9.1)	0.047
IL-1α	10.7 (7.7–18.2)	8.4 (2.3–12.6)	0.093	IL-12p70	3.0 (2.0–5.4)	4.8 (0.3–9.7)	0.721
IL-4	10.4 (7.4–15.9)	8.7 (4.5–11.7)	0.241	IL-11	1.8 (1.1–3.6)	2.8 (2.1–6.9)	0.333

Cytokines are lined up in the order of their expression levels in EBC with the highest first. The cytokine order of saliva is lined up to match that of EBC.

Abbreviations: Mig, Monokine induced by IFN-γ, IP-10, IFN-γ-in-ducible protein 10; MIP, Macrophage inflammatory protein; IL-6sR, IL-6 soluble receptor; M-CSF, macrophage colony-stimulating factor; PDGF, platelet-derived growth factor. Values are median (interquartile range).

very small and that the main contributor to EBC cytokines would be ELF.

The influence of salivary contamination on EBC has been examined in several studies (Griese et al. 2002; Gaber et al. 2006; Horvath et al. 2005; Mutlu et al. 2001; Effros et al. 2002). Amylase activity was once recommended to be measured routinely for monitoring the salivary contamination. However, amylase is not specific to saliva and at present the routine measurement of amylase activity is not recommended. The electrolyte ratios in saliva have been reported to differ from those in EBC (Effros et al. 2002). Further, EBC has been shown to contain proteins not present in saliva using two-dimensional gel electrophoresis (Griese et al. 2002). Thus, saliva has not been considered to be a major contributor to EBC. However, the influence of salivary contamination on the assessment of inflammatory

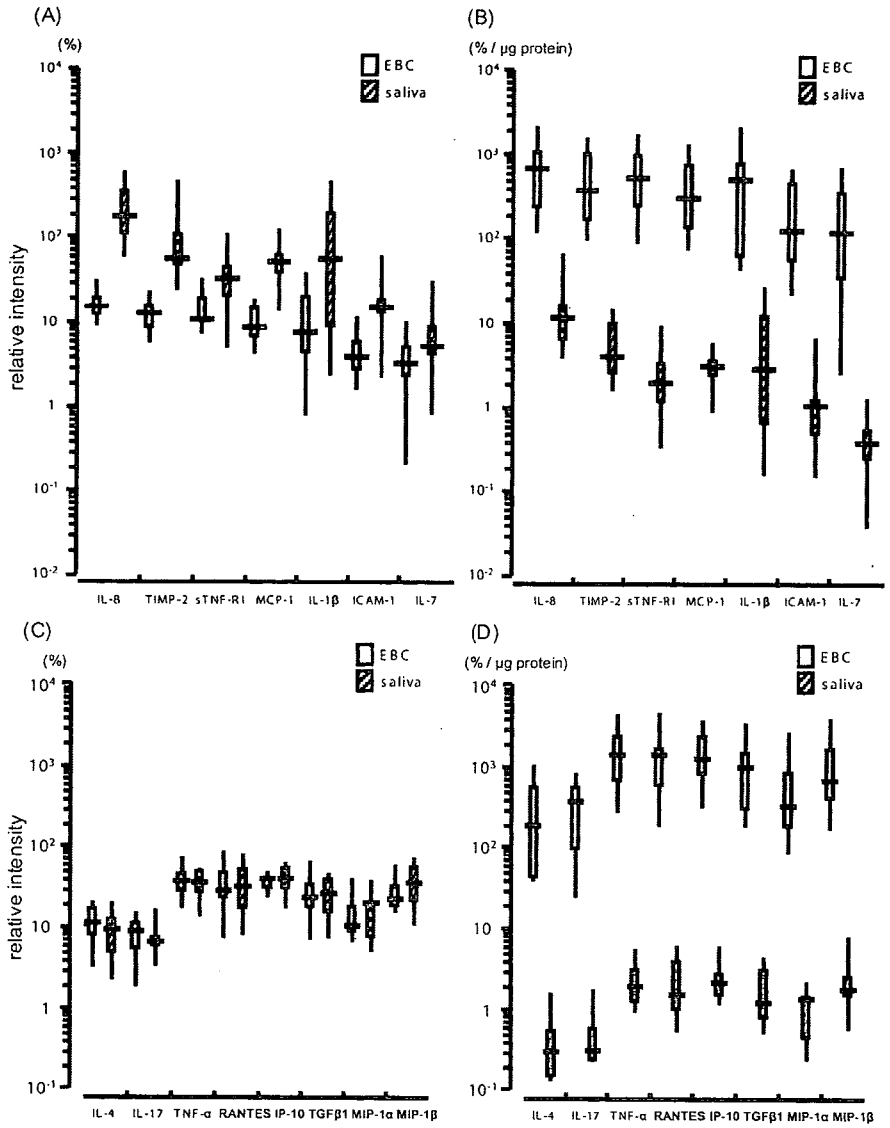

Figure 1. Cytokine expression levels of EBC and saliva before and after correction by the total protein content. The expression levels of seven cytokines more up-regulated in saliva than in EBC (IL-8, TIMP-2, sTNF-RI, MCP-1, IL-1β, ICAM-1 and IL-7) (Fig. 1A), and the expression levels of eight cytokines which have been reported to be more up-regulated in EBC from asthmatic patients than healthy controls (Fig. 1C) are shown. The expression levels of those cytokines corrected by the total protein content are shown (Figs. 1B and 1D). After protein correction, the expression levels of these cytokines were significantly higher in EBC than in saliva ($P = 0.0051$). Values are median (interquartile range).

molecules in EBC has not been fully investigated. Although Simpson JL et al. measured the IL-8 levels in EBC and saliva samples from asthmatic patients, IL-8 was not measurable in all EBC samples and the detection level was almost near the detection limit of the assay. Furthermore, the salivary influence on the IL-8 level in EBC was not assessed (Simpson et al. 2005). The salivary influence on EBC cytokines has not been adequately clarified until now.

In the present study, the expression levels of several cytokines were found to be more up-regulated in EBC than in saliva. As mentioned above, when corrected by the total protein contents, the corrected cytokine expression levels of all cytokines were significantly higher in EBC than in saliva. This suggests that EBC and saliva might have different cytokine properties and the salivary influence on EBC cytokine might be negligibly small. A cytokine protein array is a semiquantitative

assay and the precise contribution of saliva is still unclear. However, it has been shown that the relative levels obtained by the protein array correlated well with the actual levels obtained by quantitative assays in several previous reports (Huang et al. 2002a; Huang et al. 2002b; Lin et al. 2003; Turtinen et al. 2004) and our results should therefore be conducive to the standardization of EBC collection.

Protein has been used as a dilution marker for EBC and BAL assessment (Jackson et al. 2007). As described in that report, protein may not be an ideal marker as a dilution parameter, but the protein can be readily measured with a small sample volume. In the present study, the sample volume of ELF was too low (10~20 μl) and, therefore, we used it as a dilution marker of EBC. Although various markers such as conductivity, electrolyte and glucose have been measured to estimate the dilution of EBC, currently there is no definitive recommendation. The values of the total protein concentration of EBC from the present study groups were similar to those of previous reports (Effros et al. 2002; Effros et al. 2003). However, the values may be affected by the collecting device or breath condenser coatings. The concentrations of cysteinyl-Leukotriens and eotaxin were significantly higher in EBC collected by ECoScreen than in that by RTube (Soyer et al. 2006). The albumin and 8-isoprostane concentrations of EBC differed among five condenser coatings (Alfaro et al. 2007). Significant differences in the thromboxane A(2) (TXA2) concentration of EBC and the detection rate of TXA2 in EBC were found between enzyme immunoassay and radioimmunoassay (Huszar et al. 2005). Consider these technical effects on the EBC protein levels and further study will be needed for the standardization of EBC assessment.

The lower airways have been considered as the dominant source of non-volatile substances in EBC. The dilution of EBC has been estimated as approximately 1:2000~1:20000 by comparing the conductivity and electrolyte concentration in EBC with those in plasma (Effros et al. 2002; Effros et al. 2003). The glucose concentration has been measured in EBC to estimate the glucose concentration in respiratory fluid, although the glucose level in EBC is affected by the underlying disease such as diabetes and lung disease (Baker et al. 2007). However, a direct comparison of the solute concentrations between EBC and the lower airway ELF has not been performed yet. Our report is the first study in which a direct comparison between EBC and ELF were performed although samples were obtained from different groups. In the present study, the mean total protein concentration of ELF was found to be approximately 3,000 times higher than that of EBC, which suggests that the dilution of EBC could be estimated as 1:3000. In addition, the mean total protein concentration of ELF was six times higher than that of saliva. This indicates that the extent to which saliva would contribute to EBC should be very small compared with ELF. Our result supports the hypothesis that the dominant source of non-volatile substances in EBC is the lower airway (Horvath et al. 2005) and previous reports that inflammatory molecule analysis of EBC is useful for monitoring the asthmatic airway condition (Shaid et al. 2002; Ko et al. 2006; Matsunaga et al. 2006; Simpson et al. 2005).

In this study, we used the mean values of the total protein concentrations of EBC and ELF from different groups to roughly estimate the dilution of EBC and the expression levels of ELF, although there are some methodological problems. We didn't perform a bronchoscopic examination for asthmatic patients because such an examination could induce an asthma attack. Although the actual cytokine expression levels of ELF should have been measured, the sample volume of ELF was too low to be applied for a cytokine array assay. Furthermore, biomarkers such as 8-isoprostane, NOx, and H_2O_2 in EBC were reported to be not correlated with those in bronchoalveolar lavage (BAL) (Jackson et al. 2007). However, BAL is generally accepted as a sampling of only the smaller airways and alveoli. In this study, ELF was obtained from the larger airway (the right or left main bronchus). We previously reported that the albumin concentration of ELF obtained from COPD patients showed a strong correlation with $\%FEV_1$ and there was no significant difference in the albumin levels according to the smoking status and age (Minakata et al. 2005). This suggests that the origin of EBC might be the central airways. Although subjects of ELF group in this study were significantly older than those of the asthmatic group, there was no difference in the degree of airflow limitation between the asthma and ELF groups. Thus, the mean total protein concentrations of ELF from the ELF group can be expected to be similar to those of the asthmatic group. Accordingly, we expect our result will be useful for future EBC studies despite its rough estimation.

In conclusion, the cytokine expression patterns of EBC and saliva were found to be different and the source of cytokines in EBC should be those

from the lower airway. This suggests that the salivary influence on the assessment of EBC cytokines would be negligibly small.

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References

Alfaro, M.F., Walby, W.F., Adams, W.C. et al. 2007. Breath condensate levels of 8-isoprostane and leukotriene B4 after ozone inhalation are greater in sensitive versus nonsensitive subjects. *Exp. Lung. Res.*, 33:115–33.

Baker, E.H., Clark, N., Brennan, A.L. et al. 2007. Hyperglycemia and cystic fibrosis alter respiratory fluid glucose concentrations estimated by breath condensate analysis. *J. Appl. Physiol.*, 102:1969–75.

Barnes, P.J., Chowdhury, B., Kharitonov, S.A. et al. 2006. Pulmonary biomarkers in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, 174:6–14.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72:248–54.

Cap, P., Chladek, J., Pehal, F. et al. 2004. Gas chromatography/mass spectrometry analysis of exhaled leukotrienes in asthmatic patients. *Thorax*, 59:465–70.

Effros, R.M., Hoagland, K.W., Bosbous, M. et al. 2002. Dilution of respiratory solutes in exhaled condensates. *Am. J. Respir. Crit. Care Med.*, 165:663–9.

Effros, R.M., Biller, J., Foss, B. et al. 2003. A simple method for estimating respiratory solute dilution in exhaled breath condensates. *Am. J. Respir. Crit. Care Med.*, 168:1500–5.

Effros, R.M., Dunning, M.B., Biller, J. et al. 2004. The promise and perils of exhaled breath condensates. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 287:L1073–80.

Effros, R.M., Casaburi, R., Su, J. et al. 2006. The effects of volatile salivary acids and bases on exhaled breath condensate pH. *Am. J. Respir. Crit. Care Med.*, 173:386–92.

Gaber, F., Acevedo, F., Delin, I. et al. 2006. Saliva is one likely source of leukotriene B4 in exhaled breath condensate. *Eur. Respir. J.*, 28:1229–35.

Gessner, C., Scheibe, R., Wotzel, M. et al. 2005. Exhaled breath condensate cytokine patterns in chronic obstructive pulmonary disease. *Respir. Med.*, 99:1229–40.

Griese, M., Noss, J. and von Bredow, C. 2002. Protein pattern of exhaled breath condensate and saliva. *Proteomics*, 2:690–6.

Horvath, I., Hunt, J., Barnes, P.J. et al. 2005. Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur. Respir. J.*, 26:523–48.

Huang, R.P., Huang, R., Fan, Y. et al. 2001a. Lin Y. Simultaneous detection of multiple cytokines from conditioned media and patient's sera by an antibody-based protein array system. *Anal. Biochem.*, 294:55–62.

Huang, R.P. 2001b. Detection of multiple proteins in an antibody-based protein microarray system. *J. Immunol. Methods*, 255:1–13.

Hunt, J. 2002. Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease. *J. Allergy Clin. Immunol.*, 110:28–34.

Huszar, E., Szabo, Z., Jakab, A. et al. 2005. Comparative measurement of thromboxane A2 metabolites in exhaled breath condensate by different immunoassays. *Inflamm. Res.*, 54:350–5.

Jackson, A.S., Sandrini, A., Campbell, C. et al. 2007. Comparison of biomarkers in exhaled breath condensate and bronchoalveolar lavage. *Am. J. Respir. Crit. Care Med.*, 175:222–7.

Kharitonov, S.A. and Barnes, P.J. 2001. Exhaled markers of pulmonary disease. *Am. J. Respir. Crit. Care Med.*, 163:1693–722.

Kharitonov, S.A. and Barnes, P.J. 2004. Effects of corticosteroids on noninvasive biomarkers of inflammation in asthma and chronic obstructive pulmonary disease. *Proc. Am. Thorac. Soc.*, 1:191–9.

Ko, F.W., Lau, C.Y., Leung, T.F. et al. 2006. Exhaled breath condensate levels of eotaxin and macrophage-derived chemokine in stable adult asthma patients. *Clin. Exp. Allergy*, 36:44–51.

Lin, Y., Huang, R., Cao, X., Wang, S.M. et al. 2003. Detection of multiple cytokines by protein arrays from cell lysate and tissue lysate. *Clin. Chem. Lab. Med.*, 41:139–45.

Liu, J. and Thomas, P.S. 2005. Exhaled breath condensate as a method of sampling airway nitric oxide and other markers of inflammation. *Med. Sci. Monit.*, 11:MT53–62.

Matsunaga, K., Yanagisawa, S., Ichikawa, T. et al. 2006. Airway cytokine expression measured by means of protein array in exhaled breath condensate: correlation with physiologic properties in asthmatic patients. *J. Allergy Clin. Immunol.*, 118:84–90.

Minakata, Y., Nakanishi, M., Hirano, T. et al. 2005. Microvascular hyperpermeability in COPD airways. *Thorax*, 60:882.

Montuschi, P., Kharitonov, S.A., Ciabattoni, G. et al. 2000. Exhaled 8-isoprostane as a new non-invasive biomarker of oxidative stress in cystic fibrosis. *Thorax*, 55:205–9.

Mutlu, G.M., Garey, K.W., Robbins, R.A. et al. 2001. Collection and analysis of exhaled breath condensate in humans. *Am. J. Respir. Crit. Care Med.*, 164:731–7.

Sack, U., Scheibe, R., Wotzel, M. et al. 2006. Multiplex analysis of cytokines in exhaled breath condensate. *Cytometry A*, 69:169–72.

Shahid, S.K., Kharitonov, S.A., Wilson, N.M. et al. 2002. Increased interleukin-4 and decreased interferon-gamma in exhaled breath condensate of children with asthma. *Am. J. Respir. Crit. Care Med.*, 165:1290–3.

Silkoff, P.E., Erzurum, S.C., Lundberg, J.O. et al. 2006. ATS workshop proceedings: exhaled nitric oxide and nitric oxide oxidative metabolism in exhaled breath condensate. *Proc. Am. Thorac. Soc.*, 3:131–45.

Simpson, J.L., Wood, L.G. and Gibson, P.G. 2005. Inflammatory mediators in exhaled breath, induced sputum and saliva. *Clin. Exp. Allergy*, 35:1180–5.

Smith, P.K., Krohn, R.I., Hermanson, G.T. et al. 1985. Provenzano MD, et al. Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, 150:76–85.

Soyer, O.U., Dizdar, E.A., Keskin, O. et al. 2006. Comparison of two methods for exhaled breath condensate collection. *Allergy*, 61:1016–8.

Standardization of Spirometry, 1994 Update. American Thoracic Society. *Am. J. Respir. Crit. Care Med.*, 152:1107–36.

The official statement of the American Thoracic Society, 1986. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am. Rev. Respir. Dis.*, 136:225–44.

Turtinen, L.W., Prall, D.N., Bremer, L.A. et al. 2004. Antibody array-generated profiles of cytokine release from THP-1 leukemic monocytes exposed to different amphotericin B formulations. *Antimicrob. Agents Chemother.*, 48:396–403.

Yamazaki, K., Ogura, S., Ishizaka, A. et al. 2003. Bronchoscopic microsampling method for measuring drug concentration in epithelial lining fluid. *Am. J. Respir. Crit. Care Med.*, 168:1304–7.

The Influence of Free 3-Nitrotyrosine and Saliva on the Quantitative Analysis of Protein-Bound 3-Nitrotyrosine in Sputum

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Abstract

Background: We have recently developed a new technique for quantitatively measuring protein-bound 3-nitrotyrosine (3-NT), a footprint of nitrosative stress, utilizing high-performance liquid chromatography with an electrochemical detection (HPLC-ECD) system. Using this system, we showed that 3-NT formation was upregulated in the sputum of both COPD and asthmatic patients. However, in order to improve the accuracy of the measurement system, We have to resolve some problems which were the influence of free amino acid form of 3-NT and of salivary contamination.

Objectives: We initially investigated the amount of the free amino acid form of 3-NT in induced sputum and compared with that of protein-bound 3-NT. Next, we evaluated the concentration of protein-bound 3-NT in saliva and compared with that in induced sputum by means of HPLC-ECD.

Methods: Five male COPD patients were enrolled. Induced sputum and saliva were obtained from the patients. The free amino acid form of 3-NT in sputum and saliva was measured by HPLC-ECD, and the protein-bound 3-NT and tyrosine in sputum and saliva were enzymatically hydrolyzed by *Streptomyces griseus* Pronase and measured for the protein hydrolysate by HPLC-ECD.

Results: The mean value of the amount of protein-bound 3-NT was 65.0 fmol (31.2 to 106.4 fmol). On the other hand, the amount of the free amino acid form of 3-NT was under the detection limit (<10 fmol). The levels of both 3-NT (sputum: 0.55 ± 0.15 pmol/ml, saliva: 0.02 ± 0.01 pmol/ml, $p < 0.01$) and tyrosine (sputum: 0.81 ± 0.43 μmol/ml, saliva: 0.07 ± 0.04 μmol/ml, $p < 0.01$) in saliva were significantly lower than in sputum. The percentage of 3-NT in saliva to that in sputum was about 3.1%, and that of tyrosine was about 9.0%.

Conclusion: The free amino acid form of 3-NT does not affect the measurement of protein-bound 3-NT. Furthermore, the influence of salivary contamination on the measurement of protein-bound 3-NT in induced sputum by means of HPLC-ECD was very small and could be negligible.

Keywords: 3-nitrotyrosine; high-performance liquid chromatography; free amino acid; saliva; induced sputum.

Introduction

Inflammation of the airways seems to plays an important role in the pathogenesis of chronic obstructive pulmonary disease (COPD) (National Institutes of Health, Updated 2005; Barnes, 2002). However, the pathogenesis has not yet been fully elucidated. Reactive nitrogen species (RNS) may be involved in the pathophysiology of the inflammatory process in COPD (Ichinose et al. 2000; Barnes, 2000). RNS are formed from the reaction of nitric oxide (NO) and superoxide anion (Beckman et al. 1990), or via the H_2O_2/peroxidase-dependent nitrite oxidation pathway (Eiserich et al. 1998).

The production of RNS was reported to be upregulated in the airways of asthmatic patients (Saleh et al. 1998; Hamid et al. 1993). Recently, we reported that the production of RNS was also upregulated in the COPD airways based on immunostaining for 3-nitrotyrosine (3-NT), which is a footprint of nitrosative stress (Ichinose et al. 2000). Since this method was semi-quantitative, we developed a new technique for the quantitative measurement of 3-NT which utilizes high-performance liquid chromatography with

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an electrochemical detection (HPLC-ECD) system. Using this system, we reported that the protein-bound 3-NT levels were increased in the induced sputum of both COPD and asthmatic patients, and there was a significant correlation between 3-NT/tyrosine value and % predicted forced expiratory volume in one second (FEV_1) in COPD patients but not in asthmatic patients (Sugiura et al. 2004). However, there are some problems to be resolved to improve the accuracy of the measurement system.

We have focused on the measurement of protein-bound 3-NT (Sugiura et al. 2004; Hirano et al. 2006) because the nitration of tyrosine residues in various proteins was reported to alter the function of the proteins (Beckman et al. 1990; Radi et al. 1991; Lipton et al. 1993; Beckman, 1996). However, The various biological samples contain both protein-bound 3-NT and the free amino acid form of 3-NT (Greenacre and Ischiropoulos, 2001), and the influence of the amount of the free amino acid form of 3-NT on the results of protein-bound 3-NT has not been assessed.

Furthermore, though the induced sputum technique by hypertonic saline inhalation that we used is an effective and relatively noninvasive method for obtaining airway secretions (Pin et al. 1992; Fahy et al. 1993), this method may have the disadvantage of a considerable and unpredictable level of salivary contamination which could influence the values of 3-NT in the induced sputum, and this issue has not been fully addressed.

Therefore, the aims of this study were to investigate the influence of the free amino acid form of 3-NT on the measurement of protein-bound 3-NT, and to evaluate the influence of saliva on the measurement of protein-bound 3-NT in induced sputum by means of HPLC-ECD in COPD patients.

Methods

Study subjects

Five male patients with COPD visiting our hospital were recruited in the current study after providing written informed consent. All patients were diagnosed as COPD and satisfied the definition of the Global Initiative for Chronic Obstructive Lung Disease (National Institutes of Health, Updated 2005). The study was approved by the local ethics committee. The clinical characteristics of these subjects are shown in Table 1.

Table 1. Characteristics of Study Subjects.

Sex (M/F)	5/0
Age (years)	71.6 ± 2.4
Smoking	
current/ex	5/0
pack-year	69.2 ± 20.4
FEV1 (L)	1.72 ± 0.80
FEV1/FVC (%)	45.0 ± 14.5
FEV1%predicted (%)	64.4 ± 29.5
IC (L)	2.83 ± 0.59

Definition of abbreviations: FEV1 = forced expiratory volume in one second, FVC = forced vital capacity, IC = inspiratory capacity, Data are expressed as mean ± SD

Sputum induction and saliva sampling

Sputum was induced according to the method described in previous studies (Pin et al. 1992; Hirano et al. 2006). Briefly, to prevent the bronchoconstriction that would be induced by hypertonic saline inhalation, all subjects inhaled salbutamol (400 μg) before sampling. Fifteen minutes after salbutamol inhalation, the subjects inhaled 4% hypertonic saline using an ultrasonic nebulizer (UN-701; AICA Co Ltd, Tokyo, Japan). Sputum sampling was performed every five minutes until the sputum volume was more than 1.0 ml. In order to prevent saliva contamination in the sputum, the oral cavity was rinsed out with water, and retained saliva was removed by rolling the sputum on dry gauze. Saliva was also obtained after sputum induction. Over 1.0 ml of saliva was sampled.

Sample processing

Sputum and saliva were immediately and gently treated with dithiothreitol (Oxoid Ltd, Basingstoke, Hampshire, UK) at 4 times the volume to dissociate the disulphide bonds in mucin molecules. The mixtures were centrifuged at 790 g for 5 min at 4°C and the supernatants were obtained. All supernatants were stored at −80°C until the measurement of 3-NT and tyrosine.

Preparation of protein-bound 3-NT, tyrosine, and free 3-NT

The protein-bound 3-NT and tyrosine in sputum and saliva were measured by the protein hydrolysate according to the method reported in the previous study (Sugiura et al. 2004). Briefly, a supernatant sample was centrifuged at 9000 g for 5 min to remove impurities. The supernatant sample was centrifuged again at 9000 g for 30 min

with an Ultrafree-MC centrifugal filter (Millipore Corp, Bedford, MA, USA) which can filtrate proteins of less than 10 kDa. The filtrate was prepared for the measurement of the free amino acid form of 3-NT, because the presence of protein of more than 10 kDa causes an obstruction in the HPLC-ECD column. The condensed supernatant was enzymatically hydrolyzed to liberate the 3-NT and tyrosine residues for the measurement of protein-bound 3-NT and tyrosine. Samples were mixed with a freshly prepared solution of *Streptomyces griseus* Pronase (Calbiochem, Darmstadt, Germany), which was dialyzed against phosphate buffered saline (pH 7.2) before use. The sample and pronase solution were mixed in a proportion of five to one in total protein. The pronase-treated samples were incubated at 50°C for 18 hours to hydrolyze the proteins. The hydrolysate was centrifuged at 9000 g with filtration for 30 min using an Ultrafree-MC centrifugal filter (10 kDa cut off), and then the filtrates were analyzed for 3-NT and tyrosine by HPLC-ECD. The protein concentration was determined by the Bradford method (Bradford, 1976).

Quantification of 3-NT by HPLC-ECD

The sample was subjected to a reverse phase column (C18:3 × 150 mm; Eicom, Kyoto, Japan) and eluted under isocratic conditions with 100 mM sodium phosphate buffer (pH 5.0) containing 5% methanol at a flow rate of 0.5 ml/min. The eluate was continuously applied to the analytical electrochemical system that consisted of two electrochemical cells. The upstream electrochemical cell was coulometric and made of porous carbon. 3-NT was reduced into 3-aminotyrosine on this cell at a reduction potential of –900 mV. The downstream cell was amperometric glassy carbon to oxidize the 3-aminotyrosine at an oxidation potential of +300 mV. The 3-NT was quantified by the response at the oxidation cell on the basis of a standard curve of electrochemical responses as a function of the authentic 3-NT (Sigma Chemical Co, St Louis, MO) concentration. The specificity of the detection of the peak for 3-NT by this system was confirmed according to the previous study and the following criteria: [1]Comparison of the retention time of the peak with that of authentic 3-NT, which is 12.9 min under these HPLC-ECD conditions, [2]Disappearance of the peak after treatment of the sample with 100 mM sodium hydrosulfate ($Na_2S_2O_4$) in PBS (pH 7.4) for 30 min at 37°C, [3] Nullification of the peak using a reduction potential of –600 mV (Sugiura et al. 2004). The values of concentration were expressed as those in original sputum or saliva.

Quantification of tyrosine by HPLC-ECD

The amount of 3-NT in each sample was standardized by the amount of tyrosine in the same sample, which was determined in another HPLC-ECD system for tyrosine. The hydrolysate was injected into a reverse phase column (4.6 × 150 mm, TSK gel ODS-80TS; Tosoh, Tokyo, Japan) and eluted to separate tyrosine under isocratic conditions with 50 mM sodium acetate buffer (pH 4.7) plus 5% methanol at a flow rate of 0.8 ml/min. The tyrosine peak was detected using an HPLC-ECD system (D-7000, Hitachi, Tokyo, Japan) and electrochemical detector (NANOSPACE, Shiseido, Tokyo, Japan). The retention time of tyrosine under these conditions is 4.1 min and showed a typical profile of electrochemical responses at +600 mV (Sugiura et al. 2004). The values of the concentration were expressed as those in original sputum or saliva.

Statistical analysis

The data are presented as mean ± SD. Mann-Whitney's U test was used to compare the concentration of 3-NT or tyrosine in sputum and saliva. A p-value of less than 0.05 was considered significant.

Results

Standard curve and detection limit

We examined the dose dependent profile of the electrochemical response for authentic 3-NT. A linear electrochemical response was observed in a wide range of authentic 3-NT concentrations (10–1000 fmol in absolute amounts). Actually, the range of 3-NT in sputum and saliva in this system was up to 100 fmol from our preliminary data. The detection limit was 10 fmol (Fig. 1).

Protein-bound and free 3-NT

We analyzed the formation of the free amino acid form of 3-NT in supernatants of sputum by using filtrates obtained with an ultrafiltration tube