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PULMONARY RESEARCH AND RESPIRATORY MEDICINE

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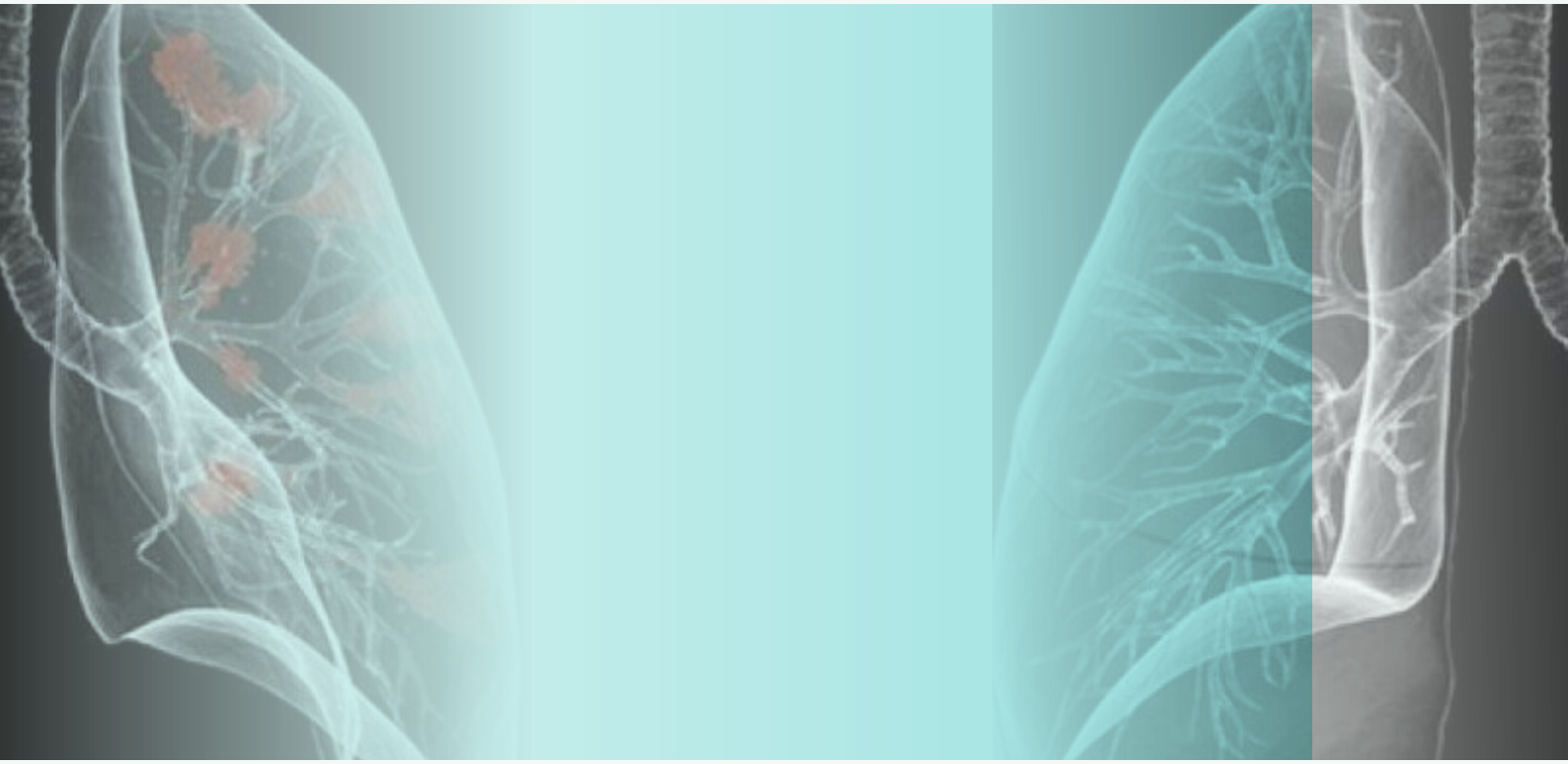

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PULMONARY RESEARCH AND RESPIRATORY MEDICINE

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PULMONARY RESEARCH AND RESPIRATORY MEDICINE

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Editorial

Corresponding author
Masahiro Kohzuki, MD, PhD
Professor and Chairman
Department of Internal Medicine and Rehabilitation Science
Disability Science
Tohoku Universiry Graduate School of Medicine
1-1, Seiryo-cho, Aoba-ku
Sendai 980-8574, Japan
Tel. 022-717-7351
Fax: 022-717-7355
E-mail: kohzuki@med.tohoku.ac.jp

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Taste Sensitivity and Nutrition in COPD Rehabilitation

Masahiro Kohzuki, MD, PhD[1*]; Satoru Ebihara, MD, PhD[2]

[1]Department of Internal Medicine and Rehabilitation Science, Tohoku University Graduate School of Medicine, Sendai, Japan

[2]Department of Rehabilitation Medicine, Toho University School of Medicine, Tokyo, Japan

Chronic Obstructive Pulmonary Disease (COPD) is a chronic disease of the lungs characterized by persistent airflow obstruction resulting from inflammation and remodeling of the airways, and may include development of emphysema.[1] Inflammatory activation in COPD induces a hypermetabolic state, characterized by catabolic and anabolic imbalance, which results in weight loss,[2] commonly seen in patients with COPD. Weight loss and low body weight are independent risk factors of morbidity and mortality in such patients.[3] One possible reason for weight loss in patients with COPD is altered taste sensitivity. Because patients with COPD may need to consume additional energy to maintain or gain weight, the taste sensory quality of meals becomes important.

Pulmonary rehabilitation is known to lead to improved exercise performance in patients with COPD. However, the relationship between pulmonary rehabilitation and taste sensitivity has not been evaluated. Therefore, we compared taste sensitivity before and after pulmonary rehabilitation in patients with COPD.[4] The six-min walk distance (6MWD), COPD assessment test, and taste test were conducted before and after 4-week comprehensive pulmonary rehabilitation. Taste sensitivity was evaluated using the filter-paper disc method for 4 taste stimuli. Taste stimuli were salty, sweet, sour, and bitter tastes. Three taste thresholds (salty, sweet and bitter) were significantly lower at the end of the PR program than at the beginning.[4] Each patient with COPD took part in the PR program, which consisted of a 20-min class one or more times a day. The exercise training consisted of walking on a treadmill, stair climbing, and ergometer cycling. Frequency of the training program was 5 times a week for 4 weeks. The intensity of the training program was 60-70% of peak work rate.[5] Following pulmonary rehabilitation, the 6MWD, COPD assessment test, salty recognition threshold, sweet recognition threshold and bitter recognition threshold improved significantly, whereas there were no significant improvements in body mass index or sour recognition threshold.[4] Pulmonary rehabilitation may improve taste sensitivity in patients with COPD.[4]

In any wasting condition, the aim of nutritional intervention is not only to treat anorexia or balance elevated energy requirements, but also to facilitate muscle protein synthesis. In the COPD field, Engelen et al.[6] was the first to show elevated whole body protein turnover in COPD and identified abnormalities in amino acids profiles in wasted COPD patients as putative therapeutic target.[7] In 2012 Cochrane meta-analysis concluded that nutritional intervention is indeed effective to improve weight, body composition and exercise performance in malnourished COPD patients.[8,9] Therefore, nutritional supplementation is an important therapeutic intervention, particularly for severely ill COPD patients with malnutrition.

Difficulties may be experienced by these COPD patients, who are struggling to breathe and eliminate CO_2 from the lungs, resulting in dyspnea, hypercapnia, hypoxia, and respiratory acidosis, which exacerbates muscle loss through oxidative stress and inflammatory responses.[10] To overcome these problems, nutritional supplements should aim to reduce metabolic CO_2 production, lower respiratory quotient, and improve lung function. Several studies have shown that high-fat supplements produce less CO_2 and have lower respiratory quotient value than high-carbohydrate supplements. In addition, high-fat supplements may be the most efficient means of providing a low-volume, calorie-dense supplement to COPD patients, and may be

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most beneficial to patients with prolonged mechanical ventilation where hypercapnia and malnutrition are most pronounced.[10]

The goal of effective COPD management is to relieve symptoms, slow disease progression, improve exercise tolerance, prevent and treat complications, and improve nutritional status and overall quality of life. In conclusion, this study suggests that a PR program may improve taste sensitivity in patients with COPD, contributing to avoiding weight loss and improving the prognosis for patients with COPD. Further studies are required to look at combining these optimal nutritional supplements with pulmonary rehabilitation for COPD patients according to their disease severity could be extremely useful and would provide a relatively cheap and simple method to improve clinical outcomes of COPD patients.

CONFLICTS OF INTEREST: None.

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Illustration

Corresponding author
Takeshi Saraya, MD, PhD
Assistant Professor
Department of Respiratory Medicine
Kyorin University School of Medicine
6-20-2 Shinkawa, Mitaka City
Tokyo 181-8611, Japan
Tel. +81 (0)422 44 0671
Fax: +81 (0)422 44 0671
E-mail: sara@yd5.so-net.ne.jp

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Marked Enlargement of Liver over a Short Period of Time

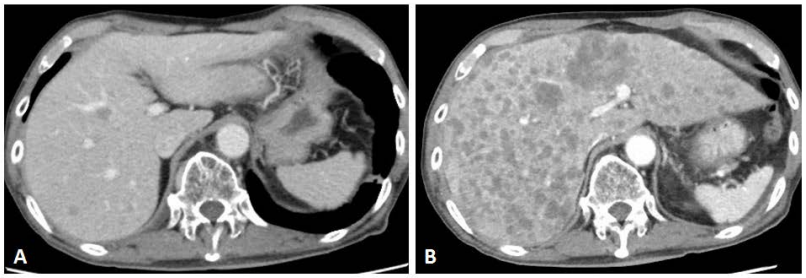
Ichiro Hirukawa, MD; Takeshi Saraya, MD, PhD*; Shoko Wada, MD; Hajime Takizawa, MD, PhD

Department of Respiratory Medicine, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka City, Tokyo 181-8611, Japan

KEYWORDS: Tumor lysis syndrome; Small cell lung cancer; Metastatic liver tumor; Liver enlargement; Short time period.

ABSTRACT

An 81-year-old man with Small cell lung cancer (limited type), who had been treated with various intravenous chemotherapies combined with irradiation during the previous year, was admitted to our hospital (day 1) for his third intravenous chemotherapy dose. Contrast-enhanced thoraco-abdominal computed tomography on day 1 demonstrated that the primary lung cancer had not grown (data not shown); however, multiple new metastatic tumors in the liver, up to 5 mm in diameter, were seen (Figure A). Two weeks later, the liver was markedly enlarged (Figure B) and repeated Computed tomography (CT). It demonstrated rapidly progressing; various sized multiple ring-enhanced tumors along with evidence of tumor lysis syndrome. On day 23, he died of multiple organ failure. Liver metastasis is a common occurrence in lung cancer patients, but the rapid enlargement of the liver over such a short time period is an extremely rare phenomenon.[1]



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Research

*Corresponding author
Mitsuo Otsuka, MD
Department of Respiratory Medicine and Allergology
Sapporo Medical University School of Medicine
South-1 West-17, Chuo-ku
Sapporo 060-8556, Japan
Tel. +81-11-611-2111 (ext. 3239)
Fax: +81-11-613-1543
E-mail: ohtsukam@sapmed.ac.jp

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Serial Measurements of Tricuspid Regurgitation Pressure Gradient by Echocardiography Predict Prognosis In Idiopathic Pulmonary Fibrosis

Yasuo Kitamura, MD[1]; Mitsuo Otsuka, MD[1*]; Gen Yamada, MD[1]; Satoshi Yuda, MD[2]; Keiki Yokoo, MD[1]; Kimiyuki Ikeda, MD[1]; Koji Kuronuma, MD[1]; Hirofumi Chiba, MD[1]; Akiyoshi Hashimoto, MD[3]; Hiroki Takahashi, MD[1]

[1]Department of Respiratory Medicine and Allergology, Sapporo Medical University School of Medicine, Sapporo, Japan

[2]Department of Cardiology, Teine Keijinkai Hospital, Sapporo, Japan

[3]Department of Cardiovascular, Renal and Metabolic Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan

ABSTRACT

Background and Objectives: Idiopathic Pulmonary Fibrosis (IPF), especially with emphysema, reportedly involved with Pulmonary Hypertension (PH). However, it is not elucidated whether pulmonary arterial pressure changes serially during the course and influence on prognosis in IPF. We examined whether serial measurements of Tricuspid Regurgitation Pressure Gradient (TRPG) by echocardiography were meaningful predictors of IPF patient survival.
Methods: We retrospectively investigated 83 IPF patients. The echocardiographic TRPG cutoff was set at 30 mmHg, and the subjects were divided into two groups: high TRPG and normal TRPG. We also evaluated the relationship between serial TRPG changes during follow-up.
Results: A total of 28 patients were included in the high TRPG group. The high TRPG group showed significantly lower %FVC and %DLco, higher A-aDO_2, shorter 6-minute walk test distance, and more frequent emphysema than the normal TRPG group. The high TRPG group also had poorer survival than the normal TRPG group. A multivariate Cox proportional hazard model demonstrated that TRPG, %FVC, and A-aDO_2 significantly affected patient survival. Thirty-six patients underwent echocardiography twice. At the time of the second echocardiography, 7 patients with normal TRPG at baseline (n=22) increased to a TRPG of more than 30 mmHg. These patients had significantly showed poorer survival.
Conclusions: TRPG is an independent prognostic factor in IPF. Emphysema involvement, decreased DLco, and decreased FVC were associated with an increase in TRPG. Serial measurements of TRPG are recommended for the early detection of PH and predict prognosis in IPF patients.

KEYWORDS: Tricuspid regurgitation pressure gradient; Idiopathic pulmonary fibrosis; echocardiography; Combined pulmonary fibrosis and emphysema.

ABBREVIATIONS: 6MWD: 6-minute walk test distance; A-aDO2: Alveolar-arterial oxygen difference; ATS: American Thoracic Society; BNP: Brain natriuretic peptide; CPFE: Combined Pulmonary Fibrosis and Emphysema; DLco: Diffusing capacity of the lung for carbon monoxide; ERS: European Respiratory Society; FEV1: Forced expiratory volume in 1 second; FVC: Forced vital capacity; HOT: Home Oxygen Therapy; IPF: Idiopathic Pulmonary Fibrosis; KL-6: Krebs vonden Lungen-6; LAA: Low Attenuation Area; LDH: Lactate Dehydrogenase; PAP: Pulmonary Arterial Pressure; PH: Pulmonary Hypertension; RHC: Right Heart Catheterization; RVSP: Right Ventricular Systolic Pressure; SP: Surfactant Protein; TRPG: Tricuspid Regurgitation Pressure Gradient.

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INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) is a progressive fibrotic disorder of unknown etiology with no cure.[1] The prediction of individual patient survival is difficult because of its heterogeneity, although the overall prognosis is poor with a median survival of 2.4-3.5 years.[2-4] Pulmonary Hypertension (PH) is an important comorbidity of advanced IPF that has a significant negative impact on survival.[5-7] Variable prevalence (range, 32%-84%) of PH has been reported.[5,6]

Combined Pulmonary Fibrosis and Emphysema (CPFE) has been proposed as a new phenotype of pulmonary fibrosis, defined by the presence of emphysema of upper lobe and fibrosis of the lower lobe.[8] PH involvement is more frequent in IPF with emphysema than in IPF without emphysema, and PH is believed to be a poor prognostic factor of CPFE.[7,8] In IPF, there is a possibility that CPFE patients are substantially included among IPF patients with PH. However, it is not clear how many CPFE patients were included in IPF with PH and related with prognosis.

Although, Right Heart Catheterization (RHC) is the gold standard for PH diagnosis,[9] this procedure is not easy to perform routinely because of its invasiveness. On the other hand, echocardiography is a noninvasive screening modality that can be useful for detecting the cause of suspected or confirmed PH.[9,10] Although echocardiography is inferior to RHC in accuracy,[11,12] several reports have suggested that it can provide a useful prognostic value of IPF.[6,13] However, most previous studies on the relationship between PH and IPF were cross-sectional analyses. Therefore, it is unknown whether clinical parameters, including Pulmonary Arterial Pressure (PAP), change serially during the natural course of IPF. The risk of PH onset is also unclear.

In the present study, we focused on PH and estimated the significance of the Tricuspid Regurgitation Pressure Gradient (TRPG), a noninvasive indicator relevant to PAP, to predict the survival of IPF patients with or without emphysema in both early stage and advanced stage disease. We also evaluated the relationship between serial changes in TRPG during follow-up and clinically practical indicators associated with the increased risk of mortality in IPF patients.

METHODS

Subjects

We performed a retrospective cohort study of 83 IPF patients at Sapporo Medical University Hospital between April 2007 and December 2013. This study was approved by the Institutional Review Board of the Sapporo Medical University Hospital. All subjects provided written informed consent. The diagnosis of IPF was made in accordance with the American Thoracic Society (ATS)/European Respiratory Society (ERS) statement.[1] All patients underwent High Resolution Computed Tomography (HRCT), pulmonary function tests, 6-minute walk tests, blood gas analysis, blood sample measurements, and echocardiography. Thirty-two of 83 patients underwent these examinations again after an appropriate interval. We excluded patients with cardiovascular diseases, infectious diseases, allergic diseases, collagen vascular diseases, granulomatous diseases, or neoplastic diseases and the patients who underwent lung operation.

HRCT and Evaluation of Emphysema

Patients were examined by chest HRCT within one month prior to echocardiography. CT scans were obtained on a Light Speed Ultra scanner (GE Health Care, Tokyo, Japan) using 1.25 mm collimation at 5 mm intervals from the sternal notch to below the diaphragm during breath-holding after a deep inspiration in a supine position at 140 kVp, 170 mA. The lungs were imaged at the window width of 1000 HU and the window level of 700 HU.

We evaluated the extent of emphysema by visual scoring in bilateral lung fields according to the method of Goddard.[14] In brief, both lungs were divided into a total of six areas consisting of three lung fields: the aortic arch, carina, and inferior pulmonary vein levels. The extent was estimated using a 5 points scale for each lesion. Total scores were calculated (maximum total: 24 points) and the severity of emphysema was graded as follows: 0 point (no emphysematous lesions), 1 point (LAA <25% of the entire lung field), 2 points 25% ≤low attenuation area (LAA) <50% of the entire lung field, 3 points (50% ≤LAA <75% of the entire lung field), and 4 points (75% ≤LAA of the entire lung field). HRCT scans were independently reviewed by 3 experienced pulmonologists. Emphysema was defined as a LAA lacking a distinct wall on HRCT. The total emphysema scores of %LAA ≥25% were categorized as IPF patients with emphysema.[15]

Pulmonary Function Tests

Patients were examined by pulmonary function tests within a month before the echocardiography. Chestac 9800 (Chest Co, Tokyo, Japan) was used for pulmonary function tests. We used parameters as follows: forced vital capacity (FVC), predicted percentage of forced vital capacity (%FVC), and forced expiratory volume one second percent (FEV_1/FVC). We measured diffusion capacity (DLco) and predicted the percentage of diffusion capacity (%DLco) according to single-breath carbon monoxide uptake. The alveolar-arterial oxygen difference (A-aDO_2) was estimated based on arterial blood gas analysis.

6-Minute Walk Test

6-minute walk test was conducted for patients according to the ATS statement, and the distance on 6-minute walk test (6MWD) was evaluated.[16]

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Echocardiography

Conventional transthoracic echocardiography was performed using Vivid7 or VividE9 (GE Health Care, Tokyo, Japan) with M5S transducer. Two-dimensional echocardiography was performed using the standard echocardiographic views, including parasternal long-axis and apical 4-, 3-, and 2-chamber views at a left lateral decubitus position. TRPG was calculated by applying the simplified Bernoulli equation: $4V^2$ (v=peak velocity of tricuspid regurgitation, m/s); and high TRPG was defined as TRPG ≥30 mmHg.[9,17]

Blood Sample Measurements

Plasma brain natriuretic peptide (BNP), surfactant protein (SP)-A, SP-D, and Krebs vonden Lungen-6 (KL-6) in sera were measured using commercially available ELISA kits at enrollment (STACIA CLEIA BNP kit, LSI medience, Tokyo, Japan; SP-A test Kokusai-F kit, SYSMEX CORPORATION, Kobe, Japan; SP-D kit YAMASA EIA II, Yamasa, Chosi, Japan; Picolumi KL-6 kit, EIDIA Co., Ltd, Tokyo, Japan).

Statistical Analysis

All data were expressed as the mean ± standard deviation (SD) or 95% confidence interval (CI). Differences between the two groups were assessed using the Mann-Whitney test. A chi-square test or Fisher's exact test was used to compare categorical data. Correlations were calculated using Spearman's correlation test. The differences between the three groups were assessed by one-way analysis of variance (one-way ANOVA). Tukey HSD post hoc tests were used for differences between each pair of groups. The survival analysis was completed according to the method of Kaplan-Meier, and the log-rank test was used to compare survival curves. The multivariate Cox's proportional hazard model was used to examine the association of selected variables with survival. Variables that were significant ($p<0.05$) in the univariate analysis were included in the multivariate model.

All tests were performed at a significant level of $p<0.05$. Analyses were completed using IBM SPSS statistics version 22 (SPSS Inc., Chicago, IL, USA).

RESULTS

IPF Patient Demographic Features

Based on echocardiographic TRPG measurements, 83 patients with IPF were classified into two groups named high TRPG (TRPG ≥30 mmHg) and normal TRPG (TRGP <30 mmHg) (Table 1). The high TRPG group included 28 patients (33.7%). They had significantly lower values of FVC, %FVC, DLco, and %DLco; higher values of A-aDO_2; and a shorter distance in 6MWD as compared with the normal TRPG group. The prevalence of emphysema in 83 IPF patients was 35% (29 of 83 patients). Emphysema was more common in the high TRPG group than in the normal TRPG group (50% *versus* 27%, $p<0.05$). There was no significant difference between the high TRPG group and normal TRPG group in terms of other demographics or serum biomarkers.

Next, we examined the relationship between TRPG and other parameters. TRPG showed significantly weak to moderate correlations with 6MWD, FVC, %FVC, FEV_1/FVC, DLco, %DLco, and A-aDO_2 (Table 2). On the other hand, no significant difference was found in the relation of TRPG with age, BNP, lactate dehydrogenase (LDH), KL-6, SP-A, and SP-D.

Prediction of Survival

Kaplan-Meier survival analysis showed that high TRPG patients had significantly worse survival than normal TRPG patients ($p=0.004$) (Figure 1).

Evaluation of Prognostic Factors

The univariate Cox's proportional hazard model demonstrated that TRPG (HR=1.095; 95% CI, 1.045-1.148; $p<0.001$) and several other variables had a statistically significant impact on survival (Table 3). The multivariate Cox's proportional hazard model demonstrated that TRPG (HR=1.059; 95% CI, 1.010-1.110; $p=0.017$), A-aDO_2 (HR=1.031; 95% CI, 1.008-1.053; $p=0.007$), and %FVC (HR=0.930; 95% CI, 0.904-0.957; $p<0.001$) significantly affected survival.

Serial Changes in TRPG during Follow-Up and Survival

Of the 83 patients, 36 underwent echocardiography twice (mean interval, 14.6±6.6 months). Among these patients, 14 and 22 were classified into the high TRPG group and the normal TRPG group, respectively, at first echocardiography. At the second echocardiographic assessment, 7 (31.8%) patients in the normal TRPG group increased to TRPG more than 30 mmHg (named "increased TRPG") (Table 4). However, the other showed TRPG less than 30 mmHg at the second assessment (named "maintained TRPG").

The increased TRPG group showed significantly lower values of FVC, %FVC, and DLco when compared with the maintained TRPG group. The rate of emphysema involvement was higher in both the increased TRPG and the high TRPG group than in the maintained TRPG group. Increased TRPG showed significantly worse survival of the maintained TRPG group ($p=0.042$) (Figure 2). Patient survival in the increased TRPG group was similar (1-year mortality: 55.6%; mean survival: 7.8 months) to that of the high TRPG group at first echocardiography (61.2%, 10.8 months; $p=0.168$).

DISCUSSION

The main purpose of the present study was to clarify whether the TRPG measurement by echocardiography was a meaningful

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	All	high TRPG	normal TRPG	*p* value	No
	(n=83)	(n=28)	(n=55)		
Sex M/F	62/21	23/5	39/16	NS	
Age	70±8.0	70±7.5	69±8.2	NS	
Smoker /never-smokers	68/15	23/5	45/10	NS	
Pack-yrs smoking	39±26	38±22	40±28	NS	
IPF specific treatment				NS	
oral corticosteroids	15	6	9		
immunosuppressant drugs	13	6	7		
Pirfenidone	27	6	21		
PH specific treatment	2	2	0		
HOT	15	8	7		
emphysema (+/-)	29/54	14/14	15/40	$p<0.05$	
6MWD (meters)	358±117	301±127	388±101	$p<0.05$	n=76
FVC (L)	2.4±0.8	2.1±0.8	2.6±0.8	$p<0.05$	n=83
FVC % pred (%)	80±24	69±24	86±22	$p<0.05$	n=83
FEV1 /FVC (L)	84±10	87±8.8	82±10	$p<0.05$	n=83
DLco (ml/min/mmHg)	9.8±3.4	7.5±2.5	11±3.3	$p<0.05$	n=72
DLco % pred (%)	47±15	36±12	50±15	$p<0.05$	n=72
A-aDO_2 (mmHg)	20±16	27±20	17±13	$p<0.05$	n=83
BNP (pg/ml)	50±76	37±27	57±90	NS	n=83
LDH (IU/l)	230±57	243±58	223±55	NS	n=83
KL-6 (U/ml)	1124±712	1289±901	1040±586	NS	n=83
SP-A (ng/ml)	78±30	81±29	77±31	NS	n=83
SP-D (ng/ml)	273±191	316±239	251±159	NS	n=83
TRPG (mmHg)	26±10	36±7.6	21±6.1	$p<0.05$	n=83

Data given as mean ± SD or numbers. IPF: Idiopathic pulmonary fibrosis; PH: pulmonary hypertension; HOT: home oxygen therapy; 6MWD: six minutes walk test distance; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; DLco: diffusing capacity of the lung for carbon monoxide; A-aDO_2: alveolar-arterial oxygen difference; BNP: brain natriuretic peptide; LDH: lactate dehydrogenase; KL-6: krebs von den lungen-6; SP: surfactant protein; TRPG: tricuspid regurgitation pressure gradient.

Table 1: The baseline characteristics at initial assessment.

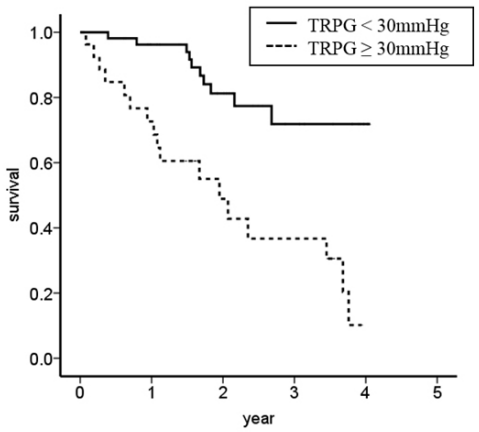

Figure 1: Kaplan-Meier survival curves for patients with IPF according to the baseline assessment of TRPG. Survival time was significantly lower in the patients with TRPG ≥30 mmHg than in those with TRPG <30mmHg (p=0.004 log-rank test).

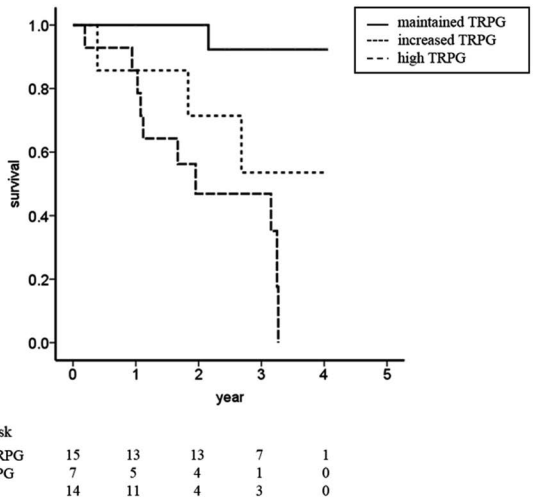

Figure 2: Kaplan-Meier survival curves for IPF patients according to the second assessment of TRPG (p=0.001 maintained TRPG *vs.* increased TRPG p=0.042, maintained TRPG *vs.* high TRPG p<0.001, increased TRPG *vs.* high TRPG p=0.168).

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	correlation coefficient	*p* value
Age	0.208	NS
6MWD (meters)	-0.296	$p<0.05$
FVC (L)	-0.284	$p<0.05$
FVC % pred (%)	-0.335	$p<0.05$
FEV1 /FVC (L)	0.239	$p<0.05$
DLco (ml/min/mmHg)	-0.32	$p<0.05$
DLco % pred (%)	-0.247	$p<0.05$
A-aDO_2 (mmHg)	0.255	$p<0.05$
BNP (pg/ml)	0.069	NS
LDH (IU/l)	0.158	NS
KL-6 (U/ml)	0.021	NS
SP-A (ng/ml)	-0.015	NS
SP-D (ng/ml)	0.074	NS

TRPG: tricuspid regurgitation pressure gradient; 6MWD: six minutes walk test distance; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; DLco: diffusing capacity of the lung for carbon monoxide; A-aDO_2: alveolar-arterial oxygen difference; BNP: brain natriuretic peptide; LDH: lactate dehydrogenase; KL-6: krebs von den lungen-6; SP: surfactant protein.

Table 2: Correlation of parameters with TRPG in 83 IPF patients.

	Parameter	HR(95% CI)	*p* value
Univariate Cox analysis	**Age**	1.012(0.965-1.062)	0.612
	Male	2.753(0.826-9.176)	0.099
	emphysema (+)	1.710(0.803-3.641)	0.164
	6MWD (meters)	0.998(0.995-1.001)	0.241
	FVC (L)	0.248(0.128-0.479)	<0.001
	FVC % pred (%)	0.920(0.894-0.946)	<0.001
	FEV1 /FVC (L)	1.136(1.069-1.207)	<0.001
	DLco (ml/min/mmHg)	0.675(0.553-0.824)	<0.001
	DLco % pred (%)	0.924(0.891-0.958)	<0.001
	A-aDO_2 (mmHg)	1.032(1.012-1.052)	0.001
	BNP (pg/ml)	0.993(0.984-1.003)	0.152
	LDH (IU/l)	0.998(0.991-1.006)	0.668
	KL-6 (U/ml)	1.000(1.000-1.001)	0.151
	SP-A (ng/ml)	0.995(0.983-1.007)	0.432
	SP-D (ng/ml)	1.002(1.000-1.003)	0.050
	TRPG (mmHg)	1.095(1.045-1.148)	<0.001
	TRPG≥30mmHg	4.510(2.058-9.881)	<0.001
Multivariate Cox analysis	**TRPG (mmHg)**	1.059(1.010-1.110)	0.017
	A-aDO_2 (mmHg)	1.031(1.008-1.053)	0.007
	FVC % pred (%)	0.930(0.904-0.957)	<0.001

IPF, idiopathic pulmonary fibrosis; 6MWD: six minutes walk test distance; FVC: Forced vital capacity; FEV1: forced expiratory volume in 1 second; DLco: diffusing capacity of the lung for carbon monoxide; A-aDO_2: alveolar-arterial oxygen difference; BNP: brain natriuretic peptide; LDH: lactate dehydrogenase; KL-6: krebs von den lungen-6; SP: surfactant protein; TRPG: tricuspid regurgitation pressure gradient.

Table 3: Prognostic factors for overall survival from initial assessment of 83 IPF patients during the follow-up period.

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	maintained TRPG	increased TRPG	high TRPG	*p* value*
	(n=15)	(n=7)	(n=14)	
observation period (days)	475 ± 198	454 ± 172	442 ± 232	NS
Sex M/F	11/4	5/2	14/0	NS
Age	66±8.8	75±3.4	70±8.9	NS
Smoker /never-smokers	12/3	6/1	31/1	NS
Pack-yrs smoking	37±27	48±26	42±22	NS
emphysema (+/-)	2/13	2/5	8/6	NS
6MWD (meters)	387±76	382±163	340±121	NS
FVC (L)	3.1±0.8	2.1±0.7	2.5±0.7	$p<0.05$
FVC % pred (%)	100±19	74±21	76±21	$p<0.05$
FEV1 /FVC (L)	77±10	81±16	85±7.5	NS
DLco (ml/min/mmHg)	13±3.2	8.4±1.5	8.0±2.7	$p<0.05$
DLco % pred (%)	60±17	44±8.8	38±12	NS
A-aDO$_2$ (mmHg)	13±7.0	23±14	21±12	NS
BNP (pg/ml)	47±60	120±208	37±26	NS
LDH (IU/l)	217±33	226±45	229±49	NS
KL-6 (U/ml)	1006±570	1047±502	1311±1111	NS
SP-A (ng/ml)	73±29	90±40	84±35	NS
SP-D (ng/ml)	218±147	275±168	361±257	NS
TRPG (mmHg)	22±3.1	25±3.4	37±5.7	NS

Data given as mean ± SD or numbers. *p values comparing maintained TRPG and increased TRPG groups. IPF: idiopathic pulmonary fibrosis; TRPG: tricuspid regurgitation pressure gradient; 6MWD: six minutes walk test distance; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; DLco: diffusing capacity of the lung for carbon monoxide; A-aDO$_2$: alveolar-arterial oxygen difference; BNP: brain natriuretic peptide; LDH: lactate dehydrogenase; KL-6: krebs von den lungen-6; SP: surfactant protein.

Table 4: Comparison of the baseline characteristics in 36 IPF patients underwent second echocardiography assessment.

predictor of IPF patient survival. We herein demonstrated that TRPG as well as FVC, DL_{CO}, and A-aDO$_2$ reflected IPF patient survival.

The precise prevalence and prognosis of PH in IPF patients remains unknown. Our study showed that 35% of IPF patients had high TRGP and demonstrated that TRPG was an independent prognostic factor for disease and patient outcomes. A previous study conducted to support the validity of our results was reported by Kimura et al.[18] Under the evaluation of 101 mild IPF patients (mean %FVC 70.2±20.1%) undergoing RHC, they showed that 35% of the patients had a mean pulmonary artery pressure (m-PAP)>20 mmHg and suggested that m-PAP is an independent prognostic factor. PH is considered to be present even in mild stages in IPF patients. Therefore, the detection of PH by echocardiography is believed to be required not only for advanced stage but also for mild stage disease.

In our study, IPF patients with high TRPG showed significantly lower %FVC than those with normal TRPG. However, FVC reportedly did not show any significant correlation with the severity of m-PAP and right ventricular systolic pressure (RVSP).[19] The discrepancy may be explained by the difference in the enrolled number of IPF patients with emphysema. CPFE patients had severely impaired DLco with preserved lung volumes, and they may have a high prevalence of PH.[8] The proportion of higher RVSP (>50 mmHg) was higher in IPF patients with emphysema than in IPF patients without emphysema.[7] In our study, IPF with emphysema were consistent with CPFE and 14 of 28 IPF patients with high TRPG were CPFE. The proportion of CPFE in IPF patients may have influenced on the correlation between FVC and PH.

We examined serial changes of TRPG and clinical parameters during patients' follow-up. Approximately 32% of the normal TRPG group experienced an increase in TRPG of more than 30 mmHg after a mean interval of 14.6 months. Song et al. reported that 9 of 36 (25%) patients with IPF but not PH at echocardiography were found to have newly developed PH during a follow-up echocardiography (mean interval of 17.7 months) and showed poor prognosis.[13] Our study also confirmed that IPF patients with increased TRPG at follow-up showed a sig-

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nificantly poorer prognosis and lower FVC and DLco at the initial examination when compared with patients who maintained TRPG. Furthermore, the IPF patients with emphysema, even in the absence of FVC decline, tended to show increased TRPG at follow-up echocardiography. Thus, TRPG may be an independent indicator that supplements routine pulmonary function tests. These results suggest the importance of monitoring at routine echocardiography through TRPG measurement in patients with IPF, particularly IPF with emphysema.

The high TRPG group showed significantly lower values in DLco and FVC, higher values in A-aDO_2, and a shorter distance in 6MWD than the normal TRPG group. Furthermore, the increased TRPG group showed significantly lower values in DLco and FVC at the initial measurement than the maintained TRPG group. Survival with increased TRPG was significantly worse and was similar to that of the high TRPG group. These results suggest that the TRPG of patients having lower values in DLco and FVC can easily increase during follow-up, even if TRPG values remain in the normal range at the initial investigation.

TRPG showed no significant association with plasma BNP levels. This result was different from previous studies wherein BNP showed a correlation with PH severity and a meaningful prediction of prognosis.[13,20,21] These studies included patients with lower mean FVC values and more severe IPF as compared with our study. Plasma BNP levels lack sensitivity in moderate PH for chronic lung disease and may be confounded by left heart abnormalities.[21] We speculated that right ventricular (RV) overload did not reflect BNP elevations in IPF patients with mild stage disease.

In addition, the serum levels of SP-A, SP-D, and KL-6 are established, useful biomarkers in IPF patients.[22-25] They are associated with rapidly declining lung function and/or poor survival. Although we hypothesized that these serum markers may be used as biomarkers of PH in IPF, we could not find a relationship between TRPG and these serum markers.

There were several limitations to our study. First, this was a retrospective cohort study and conducted at only one institute. Therefore, the number of subjects who could be examined for serial changes in TRPG was small. Second, we did not evaluate other RV function parameters. Several RV echocardiographic parameters have been associated with the prognosis of IPF with PH. Rivera-Lebron et al reported that the ratio of right ventricle to left ventricle diameter, right ventricular dilation, and tricuspid annular plane systolic excursion were associated with an increased risk of death.[26] Further studies are required to examine the relationship between the other RV parameters and IPF. Third, we did not sufficiently evaluate the HRCT findings of emphysema. In the present study, although we checked HRCT to diagnose IPF with emphysema, we did not analyze the relationship between the proportion of patients with emphysema or emphysema subtypes and TRPG. Todd et al reported that a paraseptal emphysema pattern in CPFE patients was an indicator of poor prognosis when compared with a centrilobular or mixed emphysema pattern.[27] Further studies are required to examine relationships between the proportion and subtypes of emphysema and PH in IPF patients.

CONCLUSION

TRPG was an independent prognostic factor of IPF. Particularly, as IPF with emphysema frequently involved PH, measuring TRPG serially was recommended for the early detection of PH. Our results suggest the importance of periodic measurement of TRPG by performing echocardiography during IPF patient follow-up.

AUTHOR'S CONTRIBUTIONS

YK, MO, HC and HT designed the study. SY and AH underwent Echocardiography and analyzed the data. YK, MO, GY, KY, KI, KK, HC and HT checked the diagnosis and eligibility of study subjects. YK, MO, GY, HC and HT analyzed and interpreted the data. All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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Research

*Corresponding author
Rakesh K. Mishra, PhD
Autoimmune Disease Center
The Feinstein Institute for Medical Research, 350 Community Drive
Manhasset, New York, USA
E-mail: rakeshadams@gmail.com

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Changes in Phosphoinositide Turnover in Airway Smooth Muscles and Blood Lymphocytes in Ova Sensitized Guinea Pig Model of Asthma

Rakesh K. Mishra, PhD*

Autoimmune Disease Center, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, New York, USA

ABSTRACT

Background and Objective: Bronchial asthma has been defined as a combination of reversible airway obstruction, increased airway responsiveness, and airway inflammation.
Protein kinase C (PKC) is a primary group of enzymes mediating signal transduction for a wide variety of functions in many different cell types. Its activation has been implicated in various inflammatory diseases. Since PKC activity is regulated by the metabolic pool of phosphoinositides and their turnover. The involvement of these phospholipids in allergic inflammatory responses is, however, yet to be fully determined. To evaluate the changes in phosphoinositide turnover in guinea pig model of asthma and correlation with PKC activity.
Methods: Male guinea pigs were sensitized with ovalbumin and day of initial allergen-specific immune response determined by intradermal test. Airway hyperresponsiveness was measured using a plethysmograph. Airway remodeling, content of mucin was assessed histochemically on lung sections, and bronchoalveolar lavaged fluid (BALF) cell composition. Total PKC activity, phosphoinositides were assessed in airway smooth muscles (ASM) and peripheral blood lymphocytes.
Results: Compared with control mice, OVA-challenged mice led to enhanced recruitment of inflammatory cells in bronchoalveolar lavage fluid, increases in inflammation scores, collagen accumulation, bronchial wall thickness and inflammatory cytokines. Also, we examined total PKC activity is increased in ASM and lymphocytes isolated from both groups. The levels of phosphoinositide intermediates increased further in both ASM and lymphocyte in Ova-sensitized group as compared to control group.
Conclusion: Ova exposure aggravated airway inflammation, airway remodelling, activation of inflammatory cytokines, and increases PKC activity in OVA-sensitized and -challenged mice, correlated with total phosphoinositide intermediates increased further in both ASM and lymphocyte.

KEYWORDS: Phosphoinositide; Asthma; Ova sensitized guinea pigs; Protein Kinase C (PKC).

INTRODUCTION

Asthma affects approximately 10% of the population in the United States and its prevalence has almost doubled in the past 20 years.[1,2] In India, its prevalence varies between 2.4 to 31.14% from different parts of the country.[3,4] Airway inflammation, persistent airway hyperresponsiveness (AHR) and airway obstruction are the main characteristics of asthma.[5] Onset of this disease starts with the sensitization to an allergen, followed by IgE-mediated response, mast cell degranulation, bronchoconstriction and recruitment of inflammatory cells. In addition, during progression of the disease, structural changes in the airways like; goblet cell hyperplasia, smooth muscle thickening and subepithelial and airway wall fibrosis are known to occur.[6-10]

Asthma is triggered by various stimuli including virus, environmental pollutants,

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tree and weed pollens, cold air, exercise etc.[11,12] The response of airway cells to these stimuli is mediated through activation of distinct transmembrane signaling intermediates.[13] The activation of protein kinase C (PKC) signaling pathway is one of the key players in asthma pathogenesis.[14,15] Previously, we and others have shown that inhibition of PKC reduces the activation of lymphocytes, inhibits the expression of Th2 cytokines by T lymphocytes from asthmatic patients,[16,17] abolishes airway smooth muscles constriction,[18] and inhibits the proliferation and structural changes in airway smooth muscle cells from asthmatic rats.[19] In another study, inhibition of PKC by calphostin-C, prevented proliferation of bovine tracheal smooth muscle cells following activation of mannose receptors by β-hexosaminidase and also abolished Ca^{2+}-dependent and -independent PKC activity[20] which suggests involvement of different isoenzymes of PKC.

Thus, it is evident that PKC plays a pivotal role in the pathophysiology of asthma. In most of the studies, investigations were carried out after the disease was established and there is no report available on the role of PKC in the etiopathogenesis of the disease. We, therefore, hypothesized that PKC mediated signal transduction pathway might be playing an important role in the onset of the diseases.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Animal Ethics Committee of Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India.

Study Design

The experiments were conducted on guinea pigs. The animals were sensitized by ovalbumin using standards protocol. The control group received the vehicle alone. The sensitization of the animals was checked every day by intradermal (ID) test using ovalbumin. The day, the sensitized animals showed a positive reaction for the first time, was recorded. The airway responsiveness to ovalbumin was measured by non-invasive body-plethysmography technique daily starting from on day 0 (baseline) till the day when the sensitization was observed by intradermal test for the first time (onset) and finally the day of maximum positivity. Besides it, the cytology BALF and histopathology of lung tissue was also performed to assess the airway changes in the lungs (Figure 1). These parameters established day 9 to be the day of onset of hypersensitivity and day 14 to be the day of maximum hyper-responsiveness. The animals were divided in two main groups, the control an experimental. Each group had 15 animals. Five animals from each group were taken for experiments on day 0, day 9 and day 14. Blood was collected from each animal directly from the heart for preparing the peripheral blood lymphocytes. The animals were then sacrificed by cervical dislocation. For assessment of inflammatory changes, bronchialveolar lavage fluid (BALF) was collected for cytology and lung removed and preserved for histopathological examination. For biochemical studies, the trachea and bronchi were removed and airway smooth muscles (ASM) dissected out. The ASM homogenate and lymphocyte lysate were prepared, followed by identification, characterization and quantification of phosphoinositides by thin layer chromatography (TLC), total PKC activity assay by radio-binding and histone phosphorylation methods. The data were analyzed statistically. $p<0.05$ was considered significant.

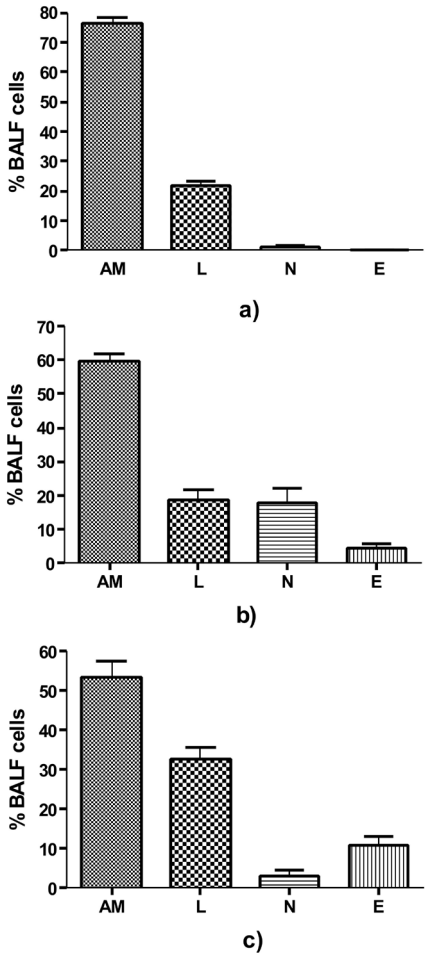

Figure 1. BALF Differential cell count (a) Control group i.e. on day 0, comprising predominantly of AM, (b) On day 9, Significant increase in N and E (p=0.002 and p=0.016 respectively) as compared to control and (c) on day 14, AM showed significant decrease to 53.20±4.23 % (p<0.0001) and a significant increase in E to 10.80±2.48%, (p=0.0001) as compared to control. AM= Alveolar Macrophages, N= Neutrophils, E = Eosinophils and L= Lymphocytes.

Chemicals and Reagents

Radioactive Chemicals: [3H] Phorbol 12, 13 dibutyrate ([3H] PDBu) (specific activity 23.5 Ci/mM), was purchased from Amersham Biosciences, UK. [32P]-γ-ATP (specific activity 4000 Ci/mM) was purchased from BRIT, Mumbai, India. Histopaque (sp. gravity 1.077), ovalbumin (Grade V), L-α phosphatidyl inositol (PI), L-α phosphatidyl inositol 4 monophospahte (PIP), L-α phosphatidyl inositol 4, 5 bisphospahte (PIP2), L-α phosphatidyl serine (PS) were purchased from Sigma Chemical Co. St Louis., USA.

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Sensitization of Guinea Pigs

The animals were sensitized with 100 µg ovalbumin and 100 mg aluminum hydroxide per ml of normal saline.[21] Control group animals received the vehicle alone. The antigen-adjuvant solution was agitated vigorously on a vortex mixer for 90 minutes and 0.5 ml injected intraperitoneally and 0.5 ml subcutaneously by dividing the amount equally among 5 different sites near the lymph nodes, viz. cervical, axillary, lumbar regions, neck and inguinal. This protocol results in sensitization of guinea pigs.

Specific Airway Conductance (SGaw)

SGaw was determined by dividing airway conductance by the lung volume (SGaw=Gaw/TGV). It was measured by non-invasive whole body plethysmography technique.[20] Briefly, the box pressure and airflow was measured (Validyne PM 18 differential transducers) and the signal amplified (CD 12 carrier demodulators). The box pressure signal was fed on the X channel and airflows on the Y channel of oscilloscope (Hewlett-Packard, USA) to obtain an X-Y plot.[22]

Collection of Broncho-Alveolar Lavage Fluid and Cytology

The bronchoalveolar lavage fluid (BALF) was collected by exposing the trachea as per the method described earlier,[23] and physiological saline (8 ml, 37 ˚C) was injected in the lung and lavaged twice. The lavage fluid obtained was centrifuged at 500 × g for 10 min at 4 ˚C. The cell pellet was given osmotic shock to eliminate the erythrocyte contamination as described by Bansal.[24] The cell suspension was centrifuged as before, pellet washed twice with saline and resuspended in 100 µl saline. The total cell count was performed using a Neubaur's Counting Chamber. Smears were stained with Giemsa stain and differential count performed on at least 300 cells in each case.

Histopathology of Lung

A histopathological study of lungs was carried out as described by Underwood et al.[25] Briefly, the lungs were removed, half the portion fixed by slowly inflating with buffered formaline and subsequently embedding in paraffin. 5 µm thick sections were stained with haematoxylin and eosin. Lung parenchyma was assessed for changes in bronchi and bronchioles. Peribronchiolar inflammation was assessed and graded on the scale of 0-3.[26] Gomori's stain for reticular fibers to demonstrate thickening of the sub-epithelial lamina reticularis and Masson Trichrome stain to demonstrate sub-epithelial collagenization were also used.

Preparation of Airway Smooth Muscles (ASM) Extract

The trachea was harvested after sacrificing the animal, and adhering extraneous material to it was carefully removed, smooth muscle portion dissected out, washed in chilled saline and soaked over a clean filter paper. The tissue was then weighed and suspended in 9 volumes of homogenizing buffer (25 mM tris HCL pH 7.5, 2 mM EGTA, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 250 mM sucrose) and homogenized in a glass homogenizer in an ice-water bath. Homogenate was then centrifuged at 700 × g for 5 min as above and supernatant used for subsequent analysis.

Preparation of Peripheral Blood Lymphocytes (PBL) Lysate

The peripheral blood lymphocytes were isolated by the method of Boyum.[27] Briefly, blood was diluted 1:1 with saline and carefully layered over histopaque (specific gravity 1.077) and centrifuged at 2000 rpm (Rotor no. 9, Rota 4R-V/FM Plasto Crafts, Mumbai, India) for 10 minutes. The opaque ring at the interface containing lymphocytes was collected, diluted with saline and centrifuged at 1500 rpm for 10 minutes. The pellet was washed twice with saline. Contaminating erythrocytes were lysed by incubating the pellet in 0.85% ammonium chloride solution. The PBL lysate was prepared by sonicating the cell suspension in the homogenizing buffer in an Ultrasonicator (Misonix Ultrasonic Processor XL- 2020) by giving five bursts of 30 sec each at an interval of one min at 0 ˚C in an ice-water bath. The cell lysate was centrifuged at 700 × g for 5 minutes and supernatant saved for subsequent analysis.

Protein Estimation

Protein contents of the ASM extracts and PBL lysate were quantified by the method of Lowry et al.[28] Equal amount of proteins from different experimental groups was used for further analyses and determinations.

Phosphoinositide Assay

Phosphoinositides were extracted and resolved by TLC as described by Bansal et al.[29] Briefly, the ASM homogenate or lymphocytes lysate (0.5 ml) in 10 mM LiCl solution containing one mg total protein was thoroughly mixed with 1.8 ml of acidified chloroformmethanol mixture ($CHCl_3$: CH_3OH: HCL; 100:200:2; v/v/v), followed by addition of 0.6 ml of chloroform and 0.6 ml of 2 M KCl. After thorough mixing, the contents were centrifuged at 500 g for 5 min at room temperature, aqueous and organic phases carefully separated and saved in separate tubes. The pellet was washed again with 2 volumes acidified chloroformmethanol mixture and aqueous and organic phases carefully separated and pooled. Organic phase containing phospholipids was dried under the stream of N2 and stored at 4 ˚C till analyzed. Phospholipids were resolved by TLC on silica gel G plates impregnated with 1% potassium oxalate and 1 mM EDTA.[30] The spots corresponding to the standards were scraped. The bands of each phosphoinositide were processed for estimation of total phosphorus by the direct gel digestion method of Misra.[31]

Protein Kinase C (PKC) Assay

PKC in ASM extracts and PBL lysate was assayed by two different methods viz. radio-ligand binding assay and histone IIIS phosphorylation as described earlier.[32]

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For radio-ligand binding assay, briefly, to 25 µl of reaction mixture (50 mM tris HCl, pH 7.5, 1 mM $CaCl_2$, 0.1% BSA, 20 mM $MgCl_2 \cdot 6H_2O$, 2 mM DTT, 5 mg/ml phosphatidyl serine (PS) and 1 µCi/ml [3H]-PDBu) in triplicate, 25 µl sample (30-50 µg protein) was added to each tube to start the reaction and contents incubated at 30 ˚C for 25 minutes. In one of the test tubes, phorbol 12-myristate 13-acetate (PMA) was added (final conc 5 µM) which served as control for the non-specific binding. The reaction was terminated by adding 2 ml chilled 10 mM tris-hydrochloride (pH 7.5). The contents were filtered on what man glass fibre filter (25 mm GF/C), washed twice with chilled 10 mM tris HCL (pH 7.5) and processed for radioactivity counting in a liquid scintillation counter (Beckman LS 6500, CA, USA). PKC activity was expressed as femtomoles [3 H]-PDBu bound per mg protein under the experimental conditions.

For histone IIIS phosphorylation assay, the standard reaction mixture (50 µl) containing 50 mM tris HCL pH 7.5, 50 µM ATP, 10 mM $MgCl_2 \cdot 6H_2O$, 100 µM $CaCl_2 \cdot 2H_2O$, 50 µg histone IIIs, 5 µg PS and 32P-γ-ATP (0.1 µCi, sp. activity 4000 Ci/mM) was kept on ice. The reaction was started by addition of 50 µl of sample containing 50 µg proteins. The tubes were vortexed and incubated for 3 minutes at 30 ˚C in a metabolic shaker. The reaction was stopped by the addition of one ml chilled 20% trichloroacetic acid (TCA). Acid precipitable material was collected on a Millipore membrane filter HA type (25 mm, 0.45 µm) under vacuum using a manifold filtration device (Millipore). The filters were washed with one ml of TCA (10%) followed by washing with 2 ml ethanol, transferring of the filters into scintillation vials containing 5 ml dioxane-based scintillation fluid and counting of radioactivity using Beckman LS 6500 liquid scintillation counter. PKC activity was expressed as femtomoles of 32P-transferred to histone IIIs per mg protein under experimental conditions

Quantitation of IgE

Total IgE in the BAL fluid was quantitated with a sandwich ELISA protocol. Briefly, the plates were coated with affinity-purified rabbit anti-IgE overnight at 4 ˚C and then blocked with 1% BSA in PBS for 1 h at 37 ˚C. The BAL fluid samples and appropriate dilutions of a standard IgE preparation were placed in the wells, and the plates were incubated for 3 h at 4 ˚C. Sample blank wells did not receive BAL fluid but were otherwise treated similarly. The bound IgE was detected with polyclonal goat anti-IgE Abs (incubation for 1 h at 37 ˚C), followed by HRP-conjugated rabbit anti-goat Abs (incubation for 1 h at 37 ˚C). The plates were developed by addition of OPD and read in an ELISA plate reader at 490 nm.

RESULTS

Ag-Specific Ige Is Present In Bal Fluid From Guinea Pigs With Allergic Airway Inflammation

IgE was detectable in BAL fluid from Ova-sensitized mice exposed to just one dose of aerosolized OVA, when the fluid was obtained 24 h after the Ag exposure. Significantly higher levels were detected after six Ag exposures, at both 3 and 24 h after the last exposure. Control mice that were sensitized to OVA but were exposed to aerosolized PBS did not show detectable levels of IgE. The levels of IgE in general correlated with eosinophilia.

Determination of Airway Hyper-Responsiveness during Onset of Immune Response

After determining the allergen specific immune response, we measured the specific airway conductance (SGaw) on days 1-14 in control and sensitized guinea pigs by whole-body plethysmography . On day 0, the SGaw of control group was 0.95±0.02 sec^{-1} cm H_2O^{-1} (Mean±SEM) and that of experimental group (immediately after sensitization) was 0.89±0.03. There was no significant change in SGaw between day 1 and 8 (data not shown) in sensitized group as compared to the control group. However, on day 9, there was >35% (36.88%±0.55%) fall in SGaw after ovalbumin inhalation in experimental group while in control group the fall was only 21.80±1.250 and the changes were statistically significant (p<0.0001), indicating that day 9 was the accurate time point of onset of the airway hypersensitivity after sensitization. On day 14, there was a further decrease (43.91%±2.131%) in SGAw (p<0.0001) as compared to control (21.14±1.60). Since there was no significant change in SGawon day 7 and 8, we concluded day 9 to be the day of onset of airway hypersensitivity.

Determination of Cytological, Pathological and Biochemical Changes during the Onset of Airway Hypersensitivity

BALF total and differential cell count was done on day 0, 9 and 14 in control and experimental group animals. The total BALF cell count in control group on day 0 was 1.9±0.14 × 106 cells/animal. In experimental group on day 0 there was no significant change, but on day 9, a significant increase in the total cell count to 3.925±0.09 × 106 cells/animal (p<0.001) and on day 14 to 6.203±0.12 × 106 cells/animal (p<0.001) was observed. On day 14, the alveolar macrophages showed further decrease to 53.20%±4.23% (p<0.0001) as compared to control. There was a significant increase in eosinophils to 10.80%±2.48%, (p=0.0001) but decrease in neutrophils to 3.30%±1.23% as compared to day 9 (p<0.003) although the number was still significantly higher (p<0.034) than that of day 0. The lymphocytes in BALF significant increased to 32.70%±3.07%, (p<0.0001) on day 14.

Histological analysis of lung sections

The histopathological changes in the proximal and distal airways were determined to assess the initiation of airway hypersensitivity. In the proximal airways of sensitized animals, an increase in thickness of airway smooth muscle and multiple areas of epithelial denudation was observed on day 14 when compared to control (Figures 2a and 2b). These changes were not observed on day 9. The distal airways revealed grade 1, peribronchiolar

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chronic lymphocytic inflammation when compared to control on day 9 (Figures 3a and 3b). On day 14, pathological changes were of grade 2 which corresponded to infiltration of lymphocytes and eosinophils in the lung (Figure 3c). Further, the onset of structural remodeling of proximal and distal airways was observed in experimental group on day 14 in which ASM hypertrophy was seen in proximal airways (Figure 4a) while distal airways showed a mild increase in thickness of the subepithelial lamina reticularis (Figures 4b, 4c and 4d) as compared to control group.

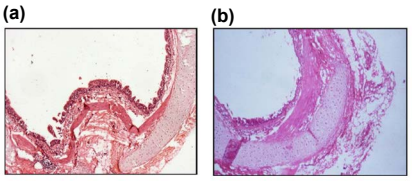

Figure 2. Pathological changes in proximal airways on day 14. H&E stain x 40. (a) Control group; showing normal histopathology and (b) Experimental group on day 14 showed epithelial denudation and airway smooth muscle hypertrophy.

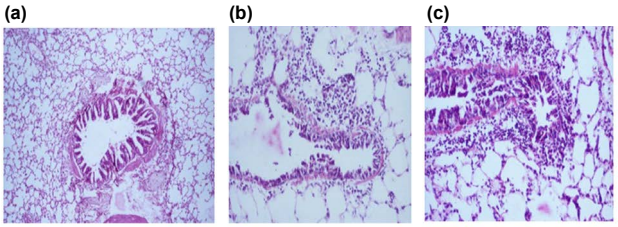

Figure 3. Distal airway inflammation, graded for peribronchiolar inflammation and percentage of eosinophilia H&E stain x 100. (a) Control group, normal bronchiole and lung parenchyma. (b) Grade 1 peribronchiolar inflammation on day 9 and (c) Grade 2 peribronchiolar inflammation on day 14, comprising of eosinophils and lymphocytes.

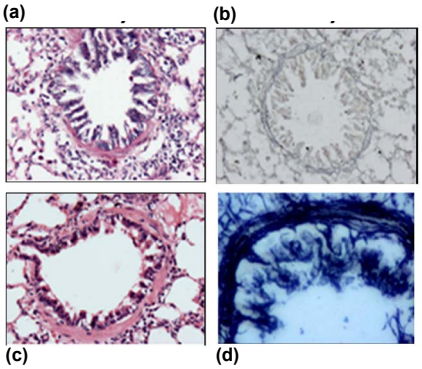

Figure 4. Structural changes in the distal airway. (a) Control group, H&E stain x 200. (b) Control group Gomori silver methenamine stain (GMS) x 200. (c) On day 14, mild increase in thickness of the subepithelial lamina reticularis, H&E stain x 200. (d) Laying down of reticulin fibres on day 14, in subepithelial region highlighted by GMS stain x 400.

PKC Activity and Expression of Its Isoenzymesin Airway Smooth Muscles (ASM) and Lymphocytes

To test our hypothesis that PKC could be a critical player during the initiation of airway hypersensitivity after sensitization, we examined the total PKC activity in ASM (Figures 5a and 5b) and lymphocytes (Figures 5c and 5d) isolated from control and ovasensitized group on days 0, 9 and 14. On day 0, there was no significant change in the PKC activity in ASM and lymphocyte as compared to control group. However, on day 9, PKC activity, measured by two different methods, increased significantly in ASM (p=0.0023 and 0.0025 by radioligand and histone III phosphorylation methods, respectively) and lymphocytes (p=0.0120 and 0.0001 respectively). On day 14, the PKC activity further increased significantly in ASM (p=0.0004 and 0.0011 respectively) as well as lymphocytes (p=0.008 and 0.0027 respectively). The increase in PKC activity on day 9 and further was >45%, suggesting physiological activation of the enzyme at the onset of the airway hypersensitivity. Since increase in the activity as well as expression of PKC coincide with the days of Ova-induced airway hypersensitivity, these results suggest that PKC could be a key mediator of initiation of airway hypersensitivity in guinea pigs.

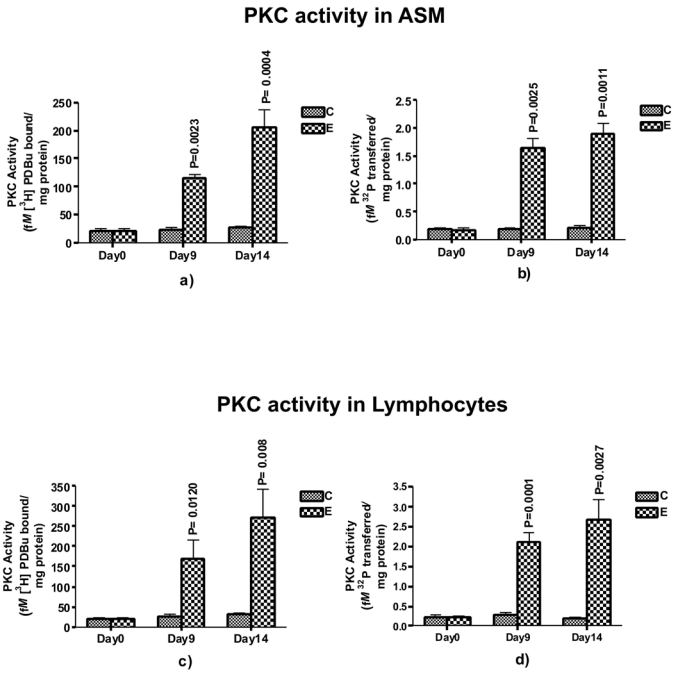

Figure 5. Assessment of PKC activity in Airway smooth muscle (ASM) and blood derived-lymphocytes. The PKC activity was measured by radio-ligand binding assay (a and c), expressed as mean±SEM femtomoles of [^{3}H] DBu bound /mg protein in ASM or per 10^6 lymphocytes and Histone phosphorylation (b, d,), expressed as mean±SEM femtomoles of ^{32}P transferred to IIIs histone per mg proteins in ASM or per 10^6 lymphocytes. The activity is shown in control and experimental groups on day 0, day 9 and day 14 representing baseline, the onset and full sensitization respectively (n=5 in each group).

Phosphoinositide turnover in Airway Smooth Muscles and Lymphocytes

Since PKC activity is regulated by the metabolic pool of phosphoinositides and their turnover, we next examined the changes in the total phosphoinositides in ASM and lymphocytes during the initiation of airway hypersensitivity. The phosphoinositides extracted and estimated were phosphatidyl inositol (PI), phosphatidyl inositol 4-monophosphate (PIP) and phosphatidyl inositol 4,5- bisphosphate (PIP2). In experimental group on day 0, there was no change in PI, PIP and PIP2 levels in ASM and lymphocytes as compared to control group (Table 1). On day 9, the level of PI increased significantly in ASM (p=0.0181) and lymphocytes (p=0.0087) as compared to control group. Similarly, the levels of PIP increased significantly in ASM (p=0.0285) and

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Day	Phosphoinositide	ASM		Lymphocytes	
		Control	Experimental	Control	Experimental
0	PI	0.328±0.028	0.310±0.019	0.336±0.047	0.293±0.002
	PIP	0.208±0.007	0.237± 0.018	0.229±0.008	0.304±0.043
	PIP2	0.367±0.022	0.305±0.056	0.354±0.020	0.336±0.054
9	PI	0.339±0.047	0.624±0.036*	0.272±0.047	0.664±0.036**
	PIP	0.240±0.045	0.415±0.008*	0.253±0.018	0.478±0.043**
	PIP2	0.312±0.009	0.516±0.057*	0.345±0.029	0.651±0.005**
14	PI	0.270±0.016	0.704±0.074*	0.358±0.041	0.862±0.014**
	PIP	0.264±0.039	0.449±0.042*	0.282±0.032	0.450±0.030*
	PIP2	0.315±0.035	0.722±0.045**	0.311±0.033	0.794±0.065*

Table 1: Phosphoinositides$^{\Psi}$ in airway smooth muscles (ASM) and lymphocytes in control and experimental groups on various days after sensitization with ovalbumin.

$^{\Psi}$Expressed as µg P_i/mg proteins (Mean±SEM), n=5 in each group
PI: Phosphatidyl inositol; PIP: Phosphatidyl inositol 4-monophosphate; PIP_2: Phosphatidyl inositol 4, 5 bisphosphate.
*p< 0.05,**<0.01

lymphocytes (p=0.0076) as compared to control group. The levels of PIP2 also increased significantly in ASM and lymphocytes (p=0.0258 and 0.0020) as compared to control group on day 9. On day 14, the levels of these phosphoinositide intermediates increased further in both ASM and lymphocytes as summarized in Table 1. These results suggest that increased activity of PKC isoenzymes during initiation of airway hypersensitivity could be associated with increased levels of phosphoinositides in ova-sensitized and challenged animal.

DISCUSSION

Asthma is a multifactorial airway inflammatory disease, characterized by airway inflammation, structural changes in the airway wall associated with progressive decline in lung function, and airway hyperresponsiveness (AHR).[5] Most of the studies on bronchial asthma on the mechanism of stimulus-induced changes have been conducted after the manifestation of the disease, which do not represent its etiopathogenesis. We, therefore, investigated the biochemical changes in airway hyper reactive guinea pig model of asthma and have presented evidence that PKC isoenzymes could be key mediators in the initiation of airway hypersensitivity during the onset of asthma. Previously, several investigators have suggested that PKC plays an active role in the progression and exacerbation of asthma.[16,17] We have also demonstrated that PKC antagonist sphingosine reduced the T lymphocytes activity isolated from asthmatic patients.[16] Further, PKC inhibitor, Ro31-8220, has been shown to inhibit the expression of Th2 cytokines from asthmatic T lymphocytes,[17] abolish airway smooth muscles constriction[18] and inhibit the proliferation and structural changes in airway smooth cells of asthmatic rats.[19] In another study, Lew et al[20] showed that calphostin-C, a PKC inhibitor, prevented proliferation of bovine tracheal smooth muscle cells following activation of mannose receptors by β-hexosaminidase and also abolished Ca^{2+}-dependent and -independent PKC activity. These evidences clearly demonstrate the role of PKC in asthma, but its role during the onset of the disease is not clearly understood. We therefore investigated the biochemical and molecular changes in airway hyper reactive guinea pig model of asthma and have presented evidence that PKC isoenzymes could be key mediators in the initiation of airway hypersensitivity during the onset of asthma.

Immunoglobulin E (IgE) plays a central role in the pathogenesis of allergic diseases, including asthma.[33,34] Allergic sensitization results from the formation of antigen-specific IgE in response to common inhalant allergens.[35,36] In our present study, on day 9 after sensitization the animals in the experimental group showed a significant positive response in i.d. which indicate that allergen-specific IgE were synthesized in enough amounts which could respond to the antigen as early as day 9. When these animals inhaled ovalbumin, they showed a significant airway hypersensitivity on day 9, suggesting that day 9 was the accurate time point for the onset of the disease. The changes were further confirmed by histopathology of the lungs, e.g. there was a significant increase in the total BALF cell counts on day 9, the first day of initiation of hypersensitivity in experimental group, which further increased (3.5fold) on day 14, similar to the findings of Sakai et al.[37]

PKC is one of the key enzymes that mediate inflammatory signals.[38] It represents a structurally homologous group of 12 isoforms which are divided into three sub-groups that differ in their cofactor requirements. These are conventional(c) PKC isoforms (α, β_I, β_{II} and γ) that require Ca^{2+} and diacylglycerol (DAG) for activation, novel (n) PKC isoforms (δ, ε, ζ, θ and µ) that are Ca^{2+} independent but require DAG and the atypical (a) PKC isoforms, namely ζ, ι and λ (the mouse homologue of human PKCι), that do not require Ca^{2+} or DAG.[14,39]

One of the major signaling pathways that involves PKC is the hydrolysis of membrane PIP_2 by phospholipase C (PLC) that generates IP_3 and DAG,[14,40] which then activate PKC.[40] Activated PKC then phosphorylates downstream signaling protein kinases leading to activation of transcription factors such as NF-kB which in turn transcribe many inflammatory genes result-

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ing in inflammatory response and pathophysiological changes in affected cells. In views of this, the role of PKC isoenzymes is crucial in the activation of inflammatory signals. PKC activity is influenced by the metabolic pool of phosphoinositides and their turnover.[41] The increase in phosphoinositides pool may be due to their increased biosynthesis or decreased breakdown by the phosphomonoesterases. The phosphoinositides are metabolized by two different pathways. In one pathway activation of phosphoinositide- specific phospholipase C (PLC) leads to the generation of diacylglycerol (DAG) and inositol triphosphate (IP_3) which act as second messengers and activate PKC.[42] In the other pathway, phosphatidyl inositol -3 kinase (PI-3K) phosphorylates and converts the inositol of various phosphoinositides into respective phosphoinositide 3 phosphate species,[41,42] the levels of which are regulated by specific phosphatases. We observed that the total contents of the phosphoinositides (PI, PIP, and PIP_2) were significantly increased on day 9 after Ova-sensitization, which coincided with significantly increased antigen-specific immune response and airway hypersensitivity. The increase in the levels of PIs was associated with corresponding increase in the total PKC enzyme activity in airway smooth muscles and blood lymphocytes. These changes were further confirmed by the histopathological changes of the distal airways which showed lymphocytic infiltration (pathological grade 1), suggesting that increased activity and expression of PKC could lead to the activation of T cells and play a pivotal role in the pathophysiology, specifically during initiation of asthma. The increased PKC α activity on day 9 suggests the activation of conventional PKCs which require calcium and DAG for their activation.[39] It is known that increase in intracellular calcium is transient and tightly regulated. After being released from intracellular pools, the level of calcium returns to normal rather quickly,[40] suggesting that initiation of airway hypersensitivity and inflammation on day 9 could involve activation of PKC α, which probably trigger the onset of the disease. However, for long term sustenance of the pathology as in chronic asthma, calcium-independent mechanism will be required.[40] Ono et al have suggested that novel PKCs do not require calcium for their activation and have been implicated in the progression of asthma.[43] The significantly increased expression of PKCε by day 14, when the immune response was fully established, as evident by maximum i.d. positivity and airway hypersensitivity, indicates that sustained activation of novel PKCs in ASM and lymphocytes could be responsible for the progression of the disease. Since in our study there was no change in the expression of PKC-τ on day 9, it could be stipulated that atypical PKCs, whose activation is independent of calcium and/or DAG, do not play any significant role in the onset of the airway inflammation.[39] These findings suggest that the onset of airway inflammation requires Ca^{2+} and DAG initially and activation of DAG-dependent mechanism in the later stage of asthma pathogenesis.

In summary, the results of this study suggest that PKC signal transduction pathway participates in onset of airway hypersensitivity and inflammation by regulating activation of lymphocytes and ASM. Therefore, the use of PKC antagonist may be a promising approach towards prevention of asthma.

CONCLUSION

The study established the day 9th to be the day of onset of airway inflammation and hypersensitivity in the guinea model of ovalbumin and day 14th to be the day when the hypersensitivity is fully developed. During the onset of the disease, the activation of PKC, particularly PKCα and PKCε, mediated signal transduction pathway play a critical role in lymphocyte infiltration and onset of airway hypersensitivity, airway remodeling and asthma pathophysiology. This study is the first one which shows a direct evidence of the role of PKC mediated pathway in the onset of airway hypersensitivity and the mechanism of the etiopathogenesis of the disease in ovalbumin sensitized guinea pig model.

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Case Report

*Corresponding author
Mehmet Unlu, MD
Department of Pulmonology
Luleburgaz State Hospital
Kirklareli, Turkey
Tel. +90 507-650-7616
Fax: +90 288-413-1046
E-mail: lidokain21@hotmail.com

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A Rare Treatment Modality and its Unusual Complication: Pneumomediastinum and Subcutaneous Emphysema Following Argon Plasma Coagulation

Emre Oner, MD[1]; Mehmet Unlu, MD[2*]; Pinar Cimen, MD[3]; Pinar Kır, MD[1]; Ziya Aygun, MD[1]

[1]Department of Emergency Medicine, Ankara University School of Medicine, Ankara, Turkey
[2]Department of Pulmonology, Luleburgaz State Hospital, Kirklareli, Turkey
[3]Department of Pulmonology, Izmir Training and Research Hospital for Thoracic Medicine and Surgery, Izmir, Turkey

ABSTRACT

Argon plasma coagulation (APC) is an effective non-contact electrosurgery tool and the use of APC in interventional pulmonology is relatively recent. Successful endobronchial APC has been described in debulking malignant airway tumors, controlling hemoptysis, removing granulation tissue from stents or anastomoses, and treating various benign airway disorders. Main complications related to APC are pneumomediastinum, subcutaneous emphysema, pneumothorax, airway fire, and burned bronchoscope. These complications are reported in less than 1% of the all cases. This report describes a case of very rare complication of APC (concomitant pneumomediastinum and subcutaneous emphysema) following an attempt to relief airway obstruction caused by a lung malignancy.

KEYWORDS: Argon plasma coagulation; Complication; Pneumomediastinum; Subcutaneous emphysema.

INTRODUCTION

Argon plasma coagulation (APC) is a non-contact form of electrosurgery that utilizes ionized argon. Ionized argon is called as plasma. The argon gas, in the presence of a high-voltage electrical field, is ionized and creating a monopolar current conducted by the plasma to the target tissue. Heat energy produced by this process causes the tissue coagulation or hemostasis. The heat evaporates intracellular and extracellular water and denatures proteins, producing the coagulative and destructive effects on tissue.[1] This technique was first used with gastrointestinal endoscopy using a flexible probe in the early 1990's and was used mainly as a modality for hemostasis during polypectomy.[2] Subsequently, it has also been used in otolaryngology and dermatology.[3,4] More recently, APC has been successfully used during bronchoscopic procedures to debulk malignant airway tumours, control hemoptysis, remove granulation tissue from stents or anastomoses, and treat a variety of other benign disorders.[5-9] Pneumomediastinum, subcutaneous emphysema, pneumothorax, airway fire, and burned bronchoscope are complications of APC. However, these complications are reported in less than 1% of the all cases.[5]

This report describes a case of very rare complication of APC (concomitant secondary pneumomediastinum and subcutaneous emphysema) following an attempt to relief airway obstruction caused by a malignant lung tumour.

CASE REPORT

A 48-year-old male was admitted to the emergency department of our hospital. He was suffering

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from stage 4 squamous cell lung cancer diagnosed approximately 2 years ago. The patient had a history of chemotherapy with cisplatinum and gemcitabine for 4 cycles which was followed by 4 cycles of Pemetrexed because of cancer progression. Following this, the diasese again progressed and the patient started treatment with oral erlotinib (150 mg/day). From that time, dyspnea remained as a leading symptom although the patient's general clinical condition was better. He was referred to the local medical council and APC was offered to him in order to reduce dyspnea. He was informed about the procedure and informed written consent form obtained. The patient tolerated the procedure well and consistently experienced relief of his obstructive symptoms. He was followed for 24 hours without any significant complication. Then, he was discharged from hospital and was advised follow-up in outpatient clinic. Three days after the APC procedure, patient presented to us with 10 hours of swelling of the neck and face.

On examination, blood pressure was 100/70 mmHg and electrocardiography showed sinus tachycardia with a rate of about 120 beats/minute. Examination of the chest revealed diminished breath sounds in the upper zone of the right lung. There were crepitus on both sides of neck. Hemogram and results of blood chemistry were within normal limits. Measurement of arterial blood gas analysis on room air revealed pH: 7.44, $PaCO_2$: 34 mmHg, PaO_2: 63 mmHg, HCO_3^-: 24 mmol/L and SaO_2: 92%, compatible with hypoxemic respiratory failure. Chest radiograph findings included a homogenous and regularly-shaped dense shadow with volume loss of the right upper lobe, presence of air in the subcutaneous tissues of the neck region, and linear air shadows along the borders of trachea (Figure 1). Thus, he underwent computed tomography (CT) of the thorax and neck. CT images revealed the presence of air trapping in the mediastinum and subcutaneous tissues which confirmed the prediagnosis of pneumomediastium concomitant with subcutaneous emphysema (Figure 2). There was no sign of a pneumothorax.

The patient was hospitalized, and nasal oxygen therapy was administered during the bed rest. He was discharged on the 5th day of follow-up as his complaints disappeared. He continues to cope with symptoms of lung malignancy. He is free of all previous symptoms related to APC procedure and has no sign of a relapse.

DISCUSSION

Pneumomediastinum is the presence of air within the confines of mediastinal structures which originates from the alveolar space or conducting airways.[10] This entity was first described by

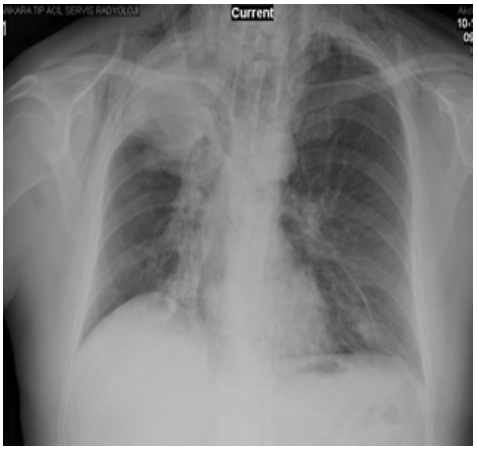

Figure 1: Chest radiograph of the patient on admission.

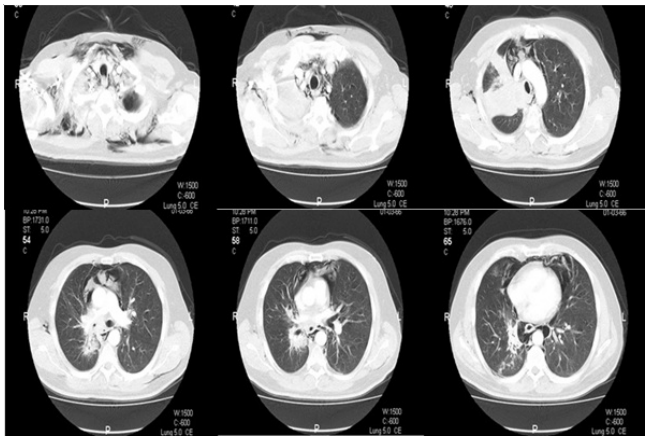

Figure 2: Thorax CT images of the patient on admission.

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Laennec in 1819.[11]

Pneumomediastium is divided into two subtypes based on etiology: spontaneous (primary) and secondary.[12] Primary pneumomediastinum is a rare medical condition without any apparent predisposing factor or disease. On the other side, the presence of air in the mediastinum is considered as secondary pneumomediastinum when a causative factor is identified, such as penetrating or blunt trauma to the chest, forceful vomiting (Boerhaave's syndrome), medical procedures such as bronchoscopy and esophagoscopy, esophageal and tracheobronchial rupture, and dental procedures. Besides, some studies reported usage of cocaine and marijuana, and the presence of asthma (usage of bronchodilators) as the secondary causes of pneumomediastinum. In this case the cause of the pneumomediastinum was flexible bronchoscopy performed for APC.

The pathophysiology of pneumomediastinum was described by Macklin and Macklin based on the results of an animal study.[13] According to their explanation, following the terminal alveolar rupture (primary pathology), alveolar air passes through the perivascular interstitial tissue towards the hilum. Then, it reaches mediastinum and is being trapped among the mediastinal structures.

Pneumomediastinum may be complicated with subcutaneous emphysema or pneumothorax in 40-100% of the cases, if intrathoracic air leaks into the adjacent soft tissues.[14] In our case it was concomitant with subcutaneous emphysema.

Chest and neck pain, dyspnea, hypotension, dysphagia, subcutaneous emphysema, and cough are the common features. Chest pain is usually retrosternal and may radiate to the neck or into the back. In almost all cases, physical examination reveals no abnormality. Palpable crepitus is only can be detected in patients complicated with subcutaneous emphysema, so it may be absent in half of the patients.[14]

The high degree of suspicion is very important for the establishment of the diagnosis.[15] There is no consensus on the investigation of this disease. Some authors point to the chest radiography (combination of posteroanterior and lateral graphs) as being sufficient in nearly all cases and CT is recommended only in doubtful cases.[16,17] However, it should be remembered that chest radiography may be normal on admission and CT is the gold standard in detecting mediastinal air. CT is also accurate in diagnosing tracheobronchial and esophageal ruptures. Electrocardiography may demonstrate non-specific ST segment changes, reduced voltage, and axis deviations in some cases.[18] In this case, posteroanterior chest radiography revealed the presence of air shadow suggesting pneumomediastinum and CT was ordered for the correction of the prediagnosis.

The treatment includes bed rest, analgesics if needed and oxygen administration. It is usually benign and non-recurrent. The patient should be hospitalized for a minimum of 24 h to prevent potential complications. In most cases, secondary pneumomediastinum resolves within several days, as seen in this case. Administration of antibiotics is only recommended in cases presented with signs of an infection or mediastinitis. However, there is also a life-threatening condition called as malignant pneumomediastinum which is characterized by the presence of excess air in the mediastinum. In such cases, subcutaneous aspiration and incisions may be required to evacuate mediastinal air, and if subcutaneous aspiration is not sufficient cervical mediastinotomy should be considered.[19]

CONFLICTS OF INTEREST

The authors have no conflict of interest to disclose. No acknowledgement, no financial or material support.

CONSENT

Written informed consent was obtained from the patient for publication of this case report and accompanying images.

AUTHOR CONTRIBUTIONS

EO: Concept and Design of the Study; Acquisition of Data; Analysis and Interpretation of Data; Revising the Article Critically For Important Intellectual Content; Final Approval of the Version to Be Published.
MU: Concept and Design of The Study; Acquisition of Data; Analysis And Interpretation of Data; Revising The Article Critically For Important Intellectual Content; Final Approval of the Version to be Published
PC: Concept And Design of the Study; Acquisition of Data; Analysis And Interpretation of Data; Revising The Article Critically For Important Intellectual Content; Final Approval of the Version to be Published.
PK: Concept And Design of the Study; Acquisition of Data; Analysis And Interpretation of Data; Revising the Article Critically For Important Intellectual Content; Final Approval of the Version to be Published.
ZA: Concept and Design of the Study; Acquisition of Data; Analysis and Iinterpretation of Data; Revising The Article Critically For Important Intellectual Content; Final Approval of The Version to be Published.

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