Effect of thromboxane A₂ synthetase inhibitor, OKY-046, on sputum in chronic bronchitis and diffuse panbronchiolitis

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ABSTRACT: The mechanisms of excessive sputum production are only partially understood. We speculated that a selective thromboxane (Tx) A₂ synthetase inhibitor, OKY-046, now used in the treatment of asthma in Japan, could decrease excess sputum production in patients with chronic airways disease.

To test this hypothesis, we carried out a double-blind, placebo-controlled study of the effects of OKY-046, administered orally at 400 mg·day⁻¹, on the sputum of patients with chronic bronchitis and patients with diffuse panbronchiolitis.

Patients treated with OKY-046 showed a significant decrease (22%) in sputum volume after 1 month, and a 39% decrease after 3 months. Although the rheological properties of the sputum and the concentrations of fucose and immunoglobulin (Ig) A in the sputum remained unchanged, significant decreases were observed in the concentrations of total protein, albumin, sialic acid and phospholipid.

Since albumin and fucose are chemical markers of plasma exudation and mucus secretion, respectively, whilst sialic acid and phospholipid are derived both from serum and mucus, our results indicate that this TxA₂ synthetase inhibitor reduced sputum volume by inhibiting microvascular leakage in the airway. OKY-046 may, therefore, be of value in the treatment of chronic bronchitis and diffuse panbronchiolitis.

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One of the major symptoms in patients with chronic obstructive pulmonary disease (COPD) is excessive sputum production, which sometimes leads to exhaustion and to the reduction of social activity.

The pathophysiological mechanisms underlying excessive production of sputum are only partially understood. Some patients with COPD produce large quantities of sputum even in the absence of respiratory infection [1]. Sputum from patients with COPD contains many different cell types, including pulmonary macrophages, neutrophils, and eosinophils [2], and these cells are capable of synthesizing and releasing phospholipid-derived mediators via the arachidonic cyclooxygenase and lipoxygenase pathways [3, 4]. It has been shown that the cyclooxygenase products of arachidonic acid stimulate both mucus [5] and water secretion [6] in the respiratory tract.

TAMAOKI *et al.* [7] demonstrated that the inhalation of 2 mL of indomethacin three times a day for 14 days decreased the amount of sputum in patients with COPD. They also showed a significant decrease in the concentrations of prostaglandin (PG) E₂, PGF_{2α}, thromboxane (Tx) B₂, and 6-oxo-PGF_{1α} in the sputum. However, to elucidate the clinical significance of these chemical mediators in airway hypersecretion, the effects of selective inhibitors to each of the mediators should be evaluated.

In our previous study, we found that the oral administration of OKY-046, a selective TxA₂ synthetase inhibitor, improved bronchial hyperresponsiveness to acetylcholine in asthmatic subjects [8]. Moreover, it significantly decreased the volume of sputum and improved nasal mucociliary clearance [9] in patients with asthma. Although these findings suggest a role for TxA₂ in sputum production in asthma, no information is available as to its effect in COPD. Accordingly, in the present study, we examined the effect of orally administered OKY-046 on sputum production in patients with chronic bronchitis and diffuse panbronchiolitis; the study was performed in a double-blind, randomized, placebo-controlled fashion. To elucidate the pathophysiological role of TxA₂ in these patients, we determined the rheological chemical properties of the sputum before and after treatment.

Methods

Patients

Thirty patients (19 males and 11 females, 36–78 yrs of age) with chronic bronchitis or diffuse panbronchiolitis, who had exhibited chronic expectoration lasting

Table 1. – Patient characteristics

Sub No.	Age yrs	Sex	Diagnosis	Skin prick test	Eos cells· μ L ⁻¹	Smoke pack-yr	FEV ₁ % pred	VC % pred	Δ FEV ₁ %	Sputum colonization
OKY-046 group										
1	67	M	CB	-ve	172	96	51	76	8.0	<i>H. influenzae</i>
2	72	F	CB	-ve	212	52	68	91	4.6	-
3	74	M	CB	-ve	409	54	54	64	6.0	-
4	39	M	CB	-ve	314	30	84	91	3.0	-
5	69	F	CB	-ve	172	29	69	79	0	<i>S. aureus</i>
6	47	M	CB	-ve	178	42	76	93	0	-
7	71	M	CB	-ve	114	75	53	67	5.8	<i>P. aeruginosa</i>
8	66	M	CB	-ve	296	47	71	66	6.0	-
9	49	F	CB	-ve	236	29	79	95	5.4	-
10	68	F	CB	-ve	97	75	58	76	0	-
11	74	M	CB	-ve	108	54	56	64	9.0	-
12	72	M	CB	-ve	46	78	55	56	3.1	-
13	41	F	DPB	-ve	32	0	75	81	2.7	<i>H. influenzae</i>
14	70	F	DPB	-ve	178	0	52	76	0	<i>P. aeruginosa</i>
15	36	F	DPB	-ve	87	0	75	78	3.0	<i>P. aeruginosa</i>
16	69	M	DPB	-ve	36	30	44	69	1.6	<i>H. influenzae</i>
Mean	62				180	43	64	76		
SEM	5				35	7	6	6		
Placebo group										
1	48	M	CB	-ve	68	30	76	96	4.6	-
2	67	M	CB	-ve	243	48	65	75	8.0	<i>H. influenzae</i>
3	52	M	CB	-ve	180	32	71	91	9.2	-
4	60	F	CB	-ve	66	32	64	81	-2.1	<i>P. aeruginosa</i>
5	46	F	CB	-ve	32	56	68	74	0	<i>H. influenzae</i>
6	78	M	CB	-ve	320	58	48	58	3.4	-
7	52	M	CB	-ve	360	60	59	68	2.4	<i>P. aeruginosa</i>
8	64	M	CB	-ve	128	62	68	97	7.0	<i>H. influenzae</i>
9	49	M	CB	-ve	190	60	79	92	0	-
10	68	F	CB	-ve	60	50	63	80	5.3	-
11	74	M	CB	-ve	48	56	56	64	0	-
12	45	M	DPB	-ve	80	0	68	77	1.1	-
13	57	M	DPB	-ve	0	0	71	85	0	<i>P. aeruginosa</i>
14	69	F	DPB	-ve	380	0	60	77	3.5	<i>P. aeruginosa</i>
Mean	59				153	38	65	80		
SEM	5				34	6	6	7		

Sub: subject; M: male; F: female; CB: chronic bronchitis; DPB: diffuse panbronchiolitis; -ve: negative; Eos: blood eosinophils; FEV₁: forced expiratory volume in one second; VC: vital capacity; Δ FEV₁: increase in FEV₁ after salbutamol; % pred: percentage of predicted; *H. influenzae*: *Haemophilus influenzae*; *S. aureus*: *Staphylococcus aureus*; *P. aeruginosa*: *Pseudomonas aeruginosa*.

for at least 3 months, were enrolled in the study after obtaining their informed written consent (table 1). The study was approved by the Ethics Committee of the hospital. All patients with chronic bronchitis satisfied the World Health Organization definition of the disease [10], and were or had been heavy smokers. Diffuse panbronchiolitis is a recently described clinicopathological entity that is found in Japanese, Chinese and Korean populations. This disorder is characterized by severe chronic airflow obstruction and excessive sputum production, accompanied by persistent sinopulmonary infection, respiratory bronchiolitis, and peribronchiolitis. Radiological findings are diffuse bronchiectasis, diffusely disseminated fine nodular shadows, predominantly in the lower lung fields, and hyperinflation of the lungs. Although

the aetiology of this disease is unknown, a genetic disorder is thought to be involved [11, 12].

The initial inclusion criteria for our study included stable disease (no episode of acute respiratory infection) for at least 4 weeks preceding the study, absence of any history of respiratory allergies, and more than 40 mL sputum daily. To assess the reversibility of the airway obstruction, the forced expiratory volume in one second (FEV₁) measurement was repeated 15 min after the inhalation of 0.2 mg of salbutamol. Patients who had more than 10% response to salbutamol on FEV₁ were excluded from this study. None of the patients had received treatment with antibiotics, mucolytic agents, or corticosteroids in the 1 month prior to the study. In 14 patients, the lungs were colonized by *Pseudomonas aeruginosa*,

Haemophilus influenzae, or *Staphylococcus aureus*, but there was no evidence of acute infection in the laboratory data or evidence of pneumonic consolidation on the chest radiograph in any patient.

Study design

The study was conducted in a double-blind, randomized, placebo-controlled fashion after a 2 week observation period. A physician (not responsible for disease follow-up or data analysis) was assigned the task of dividing the patients into two groups matched for clinical diagnosis and sputum production. The first group consisted of 16 patients who received OKY-046 200 mg *b.i.d.* for 12 weeks. The second group consisted of 14 patients who received placebo in the same manner. There was no significant difference between the two groups in age, gender, number of diagnoses and bacterial colonization (table 1). Other medications were unchanged and there was no additional treatment during the study.

Each patient recorded his/her sputum volume scores on standardized charts in the morning (5.00 a.m. to 11.00 a.m.), at noon (11.00 a.m. to 5.00 p.m.), in the evening (5.00 p.m. to 11.00 p.m.), and at night (11.00 p.m. to 5.00 a.m.) every day for 14 weeks. The use of this chart enabled patient compliance to be controlled with regard to sputum collection and daily drug intake. Scoring of the sputum volume was carried out according to the standards for evaluating the severity of adult bronchial asthma, as determined by the Japanese Society of Allergology [13], with score modification. Briefly, sputum volume was scored as two points for more than 20 mL in 6 h, one point for 1–19 mL in 6 h, and no points for no expectoration in 6 h. The patients were given plastic cups with volume marker lines for assessing the sputum volume. They were asked to swallow saliva immediately before the expectoration of sputum to minimize contamination by saliva. The sum of the daily points was 0–8, and the average number of points scored daily was calculated for 2 week periods.

Rheological analysis of sputum

Sputum samples for rheological and chemical analysis were collected during the observation period and at the end of the trial. Sputum samples were frozen immediately after expectoration and stored at -20°C until use. The samples were studied within 3 months and were not repeatedly frozen and thawed. Prior to the rheological analysis, half of the sputum samples were allowed to thaw at room temperature. The thawed sputum samples were allowed to relax for 30 min to minimize damage to the gel structure associated with the freezing process. The viscosity of sputum was measured with a rotational rheometer (Low-Share 30; Contraves, Zurich, Switzerland). The apparent viscosity was calculated after the shear stress was measured at a low shear rate (0.73 s⁻¹) at 37°C [14]. The spinability of sputum was determined with a filancemeter (Sputum Tester Type-NY;

Nihon Rheology Instruments, Funabashi, Japan). One millilitre of sputum was introduced into a reservoir and aspirated by a prehension system at a constant rate (10 mm·min⁻¹). Aspiration of the samples induced the formation of a thread. The maximum length of the thread at the moment of thread rupture was measured as an index of spinability [15].

Chemical analysis of sputum

For the chemical analysis, half of the frozen sputum samples were thawed at room temperature and then homogenized. The pH was measured with a pH-meter (D-12; Horiba, Kyoto, Japan). The total protein content was estimated by the method of LOWRY *et al.* [16], employing the folin phenol reagent. Fucose was measured with a sulphuric acid and thioglycolic acid assay [17]. Sialic acid was assessed by the thiobarbituric acid assay [18]. Albumin concentration was determined with an enzyme-linked immunosorbent assay (ELISA), employing a mouse monoclonal anti-human-albumin antibody (Sigma) as a capturing antibody (Ab) and a rabbit polyclonal Ab directed against albumin (Sigma) as a second Ab. Flat-bottomed 96-well microtitre plates (Nunc) were coated with 100 µL·well⁻¹ monoclonal mouse anti-human albumin antibody (100 µg·mL⁻¹) for 2 h at 37°C and then washed with phosphate-buffered saline (PBS). Non-specific binding sites on the microtitre plates were blocked with 10% goat serum in PBS, and the plates were then incubated for 2 h at 37°C. The plates were then rinsed with PBS, and diluted samples (100 µL) were added, followed by incubation for 2 h at 37°C. The plates were washed, 100 µL·well⁻¹ polyclonal rabbit anti-human albumin antibody (immunoglobulin G (IgG); 1 µg·mL⁻¹) was added, and plates were incubated for 2 h at 37°C. The plates were then washed five times. Diluted sheep anti-rabbit IgG conjugated with alkaline phosphatase (100 µL; Dako, Carpinteria, CA, USA) was added to each well, and the plates were incubated for 2 h at 37°C. After thorough rinsing, 100 µg·well⁻¹ p-nitrophenyl phosphate (Sigma) was added, the plates were incubated for 30 min at room temperature, and the reaction was terminated with 1 M NaOH solution. The plates were read at A405 in an ELISA reader. All assays were performed at least in duplicate.

Immunoglobulin A (IgA) levels were measured by ELISA in the manner described above, except that a mouse monoclonal anti-human IgA antibody (Sigma) was employed as a capturing antibody (Ab) and rabbit polyclonal Ab directed against IgA (Sigma) was used as a second Ab.

Phospholipid was measured enzymatically with a commercially available kit (Wako Chemicals Co., Osaka, Japan) [19].

Statistical analysis

Values were expressed as mean±SEM. Statistical comparisons of means within the same groups were performed with two-tailed paired Student's *t*-test, and for

Table 2. – Laboratory data before and after treatment

	Control group n=14			OKY-046 group n=16		
	Before		After	Before		After
VC % pred	80±7	NS	78±5	76±6	NS	77±6
FEV ₁ % pred	65±6	NS	63±5	64±6	NS	62±7
PEF L·s ⁻¹	4.5±0.7	NS	4.4±0.6	4.4±0.6	NS	4.5±0.8
WBC cells·μL ⁻¹	5760±786	NS	5980±652	5670±528	NS	5530±426
Neutrophils %	51.3±5.4	NS	52.6±4.9	50.8±5.1	NS	49.6±3.8
Eosinophils %	3.2±2.1	NS	4.2±3.5	2.6±2.2	NS	2.9±2.3
Lymphocytes %	37.8±3.1	NS	36.9±3.0	38.0±3.3	NS	39.4±3.8
Monocytes %	8.5±0.5	NS	6.2±0.6	8.4±0.4	NS	7.9±0.3
CRP mg·mL ⁻¹	0.7±0.4	NS	0.9±0.6	1.1±0.3	NS	1.0±0.6
Cold agglutinin mg·mL ⁻¹	12±6	NS	12±8	14±1	NS	13±2
IgA mg·mL ⁻¹	326±124	NS	362±156	372±130	NS	360±126

PEF: peak expiratory flow; WBC: white blood cell counts; CRP: C-reactive protein; IgA: immunoglobulin A; NS: nonsignificant. For further abbreviations see legend to table 1.

comparisons between the control group and the OKY-046 group, nonparametric Mann-Whitney U-tests were used. A p-value of less than 0.05 was considered statistically significant.

Results

Clinical assessments

Table 2 shows the laboratory data before and after treatment. Neither the placebo nor the OKY-046 treated groups showed any significant changes in percentage predicted vital capacity (VC), percentage predicted forced expiratory volume in one second (FEV₁), peak expiratory flow (PEF), white blood cell count (WBC), cell differential, C-reactive protein (CRP), or cold agglutinin during the trials. In the subjects with culture-positive sputum, there was no evidence of infection, in terms of elevated CRP levels or blood neutrophil counts, throughout the study. Administration of OKY-046 400 mg·day⁻¹ did not change sputum microbiology, which remained the same before and after treatment. No side-effects, such as bleeding tendency, were observed.

Effects of OKY-046 on sputum production

During the observation period, the baseline values for sputum volume were similar in the placebo and OKY-046 groups, *i.e.* there was no significant difference between the two groups.

As shown in figure 1 and 2, significant differences were observed in sputum volume scores between the placebo and OKY-046 groups in every 2 week period during the treatment. The placebo group did not show a significant change in the sputum volume score, while the administration of OKY-046 400 μg·day⁻¹ significantly decreased the sputum volume score from 5.7±0.3 in the observation period to 4.5±0.4 (22% reduction) one month later ($p<0.02$), 3.8±0.5 (33% reduction) two months later ($p<0.001$), and 3.5±0.5 (39% reduction) three months

later ($p<0.001$). In the OKY-046 treated group, 11 of the 16 patients showed improvement by more than 1 point in the sputum score.

Rheologic analysis of sputum

Rheological and chemical analyses were performed by a specialized examiner who had not been given any of the clinical data, including patient's names.

Previous studies have reported that purulent sputum exhibited significantly higher viscosity and lower spinability compared to mucoid sputum samples [20, 21]. In this study, we observed no significant difference before and after treatment in either the placebo or the OKY-046 group (table 3).

Fig. 1. – Time course of sputum volume score in patients with chronic bronchitis and diffuse panbronchiolitis. Values represent mean sputum scores (±SEM) for 2 weeks. Differences between placebo and OKY-046 groups were statistically analysed as described in the text. —●—: placebo control (n=14); —○—: OKY-046 (n=16). *: $p<0.05$; **: $p<0.02$, compared to placebo group.

Fig. 2. — Mean 2 week individual scores during observation period and after 3 months of treatment with OKY-046 or placebo in patients with chronic bronchitis and diffuse panbronchiolitis. ●—● : placebo group; ○—○ : OKY-046 group.

Table 3. — Rheological and chemical properties of sputum

	Control group n=14			OKY-046 group n=16		
	Before		After	Before		After
Viscosity Pa·s ⁻¹	6.3±0.28	NS	5.8±1.0	6.8±1.2	NS	6.6±0.77
Spinability mm	13.2±4.8	NS	14.3±4.2	12.5±3.8	NS	12.7±2.6
pH	7.78±0.26	NS	7.75±0.25	7.76±0.14	NS	7.82±0.14
Total protein mg·mL ⁻¹	9.9±1.3	NS	10.3±2.7	11.7±1.4	p<0.02	8.6±0.83
Albumin µg·mL ⁻¹	698±108	NS	657±116	858±114	p=0.02	595±147
Phospholipid mg·mL ⁻¹	4.33±0.46	NS	4.05±0.36	4.55±0.43	p<0.05	3.65±0.47
Sialic acid mg·mL ⁻¹	1.23±0.13	NS	1.14±0.21	1.30±0.15	p=0.05	1.08±0.18
Fucose mg·mL ⁻¹	1.26±0.06	NS	1.18±0.19	1.44±0.14	NS	1.30±0.14
IgA mg·mL ⁻¹	1.35±0.17	NS	1.29±0.24	1.49±0.16	NS	1.28±0.16

IgA: immunoglobulin A; NS: nonsignificant.

Chemical analysis of sputum

Data on the chemical properties of sputum are shown in table 3. During the observation period, there was no difference between the control and the OKY-046 groups. At the end of the treatment period (3 months later), no significant change in any component was observed in the placebo group. In the OKY-046 group, however, total protein ($p<0.02$), sialic acid ($p=0.05$), albumin ($p=0.02$), and phospholipid ($p<0.05$) had decreased significantly. No significant changes were observed for pH, fucose and IgA values after treatment compared to before treatment.

Discussion

OKY-046 is a selective thromboxane synthetase inhibitor [22] that is now available for the treatment of bronchial asthma in Japan. This study demonstrated that the inhibition of TxA₂ decreased the sputum volume in chronic bronchitis and diffuse panbronchiolitis.

The effects of drugs that act on the airway secretory

system are difficult to assess, since measurement of sputum volume is not easy in socially active patients and also because there is no absolute way of measuring the quantity of airway secretions in humans. We used a sputum volume scoring system (with score modifications) already used in patients with bronchial asthma [13] based on sputum collection in a plastic cup. By employing this method, sputum volume and drug compliance can be monitored at the same time. A sputum volume of more than 40 mL in 6 h is scored as three points. None of our patients exceeded this level throughout the study.

In this study, there was no significant difference in FEV₁ values before and after treatment. Furthermore, during the study period, no episode of acute airway infection and no significant changes in sputum microbiology and systemic inflammatory responses, in terms of values for WBC and CRP, were observed. These results suggest that OKY-046 had no direct effect on pulmonary function and sputum microbiology in these patients.

Because the rheological characteristics of mucus are the major determinant of the mucociliary transportability of sputum, we measured the viscosity and spinability of the sputum. Previous studies have reported that

the viscosity is influenced by the degree of infection [20], by deoxyribonucleic (DNA) content [23], and by lysozyme and IgA concentration [24]. The spinability of sputum is also highly dependent on the degree of purulence of the sputum [20, 21]. It has been reported that spinability is positively correlated to the mucociliary transport capacity of sputum in the frog palate, whereas viscosity is negatively correlated to this capacity [24]. In this study, OKY-046 had no direct effect on the rheological characteristics of mucus; however, the effects of the drug on mucociliary clearance should also be evaluated in terms of ciliary motion. The present study showed that OKY-046 significantly reduced sputum albumin concentration. Another study has reported that albumin agglutinated individual cilia and, consequently, destroyed the co-operative motion of the cilia [25].

Analysis of the chemical properties of sputum points to the involvement of TxA_2 in sputum production. Whilst albumin is regarded as a marker of serum-derived constituents [23], fucose is derived from the mucous secretions of the respiratory tract, and sialic acid and phospholipid are derived from both mucus and serum [23]. In this study, sputum levels of albumin, sialic acid, and phospholipid decreased, whereas the fucose level remained unchanged, suggesting that the inhibition of TxA_2 reduced microvascular leakage without reducing mucus secretion.

IgA plays a role in protecting against bacterial infection; approximately 50% or more of the IgA in airway secretion is secretory IgA, the remainder being serum IgA. Because the concentration of IgA in sputum increases in patients with acute exacerbation of chronic bronchitis, but decreases in patients with advanced disease [26], the monitoring of sputum IgA level may represent a useful marker of chronic airway inflammation. Our findings here show that no significant change occurred in sputum IgA concentration during treatment with OKY-046, suggesting that there was no remarkable change in the level of airway inflammation. The reduction of microvascular leakage may also explain the reduction in the concentration of sputum phospholipid in the OKY-046 group; however, sputum phospholipid is not a specific marker by itself, since sputum phospholipids are derived from serum, airway surfactant, and the cytoplasm of respiratory tract mobile cells [27].

It is rather surprising that the role played by chemical mediators in excessive sputum production in COPD patients has not attracted much attention, considering their effects on mucus secretion and airway microvascular leakage [28]. In fact, little is known of the effect of TxA_2 on submucosal gland secretion and goblet cell glycoprotein secretion [28]. On the other hand, TxA_2 has been shown to induce significant airway microvascular leakage. LOTVALL and co-workers [29] showed that a thromboxane mimetic, U-46619, produced plasma exudation in the airways of the guinea-pig. They also found that plasma exudation in airways induced by inhaled platelet-activating factor (PAF) was inhibited both by OKY-046 and a TxA_2 receptor antagonist, ICI-192605 [30], suggesting that the release of TxA_2 during PAF inhalation may induce plasma exudation in the airways.

The results of these experimental studies by LOTVALL and co-workers [29, 30] and our study suggest that TxA_2 plays a role in sputum production.

In conclusion, an orally administered TxA_2 synthetase inhibitor, OKY-046, decreased sputum volume, probably by inhibiting airway microvascular leakage. The results of this study point to the potential importance of TxA_2 in sputum production in patients with chronic bronchitis and diffuse panbronchiolitis, and provide another therapeutic modality in patients with excess sputum production.

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