Allergology International. 2006;55:305-309

ORIGINAL ARTICLE

Awarded Article, Annual Meeting of JSA

Reduction of Eosinophils in Small Airways by Inhaled Steroids is Insufficient in Patients with Adult Asthma

Hiroshi Tanaka[1], Midori Hashimoto[1], Masaru Fujii[1], Nobuyuki Tanaka[1], Kazuhiko Suzuki[1], Toyohiro Saikai[1] and Hiroki Takahashi[1]

ABSTRACT

Background: Recent reports suggest that small airway as well as large airway involvement in asthma is important. We investigate the therapeutic effects of a meter-dose inhaler of chrolofluorocarbon-beclomethasone dipropionate (CFC-BDP) and dry-powder fluticasone (DP-FP).

Methods: Lung specimens obtained at operation due for small size lung cancer in 16 asthmatic patients and 16 controls were evaluated immunohistochemically using antibodies of EG2 (eosinophil), AA1 (mast cell), CD68 (macrophage), and CD34 (pluripotent hematopoietic stem cell). We calculated the number of each cell type in 5 fields in the inner and outer areas of large airways (luminal diameter; $\geq$2 mm) and small airways (<2 mm) using computer software.

Results: In asthmatic patients eosinophils were significantly increased in both inner and outer areas of small airways and the number of CD34+ cells was significantly elevated in inner areas as compared with controls. Although the density of eosinophils in the inner area of large airways was significantly suppressed ($p < 0.02$), there was no such suppression in the inner areas of small airways in asthmatic patients treated with CFC-BDP or DP-FP.

Conclusions: It was speculated that inhaled CFC-BDP and DP-FP might deposit mainly in large airways and fail to fully reach small airways, consequently allowing eosinophilic inflammation to continue in small airways.

KEY WORDS

bronchial asthma, eosinophil, inhaled corticosteroid, small airways, treatment

INTRODUCTION

Asthma is characterized by eosinophil dominant chronic inflammation throughout large and small airways.[1,2] However there have been no reports on the therapeutic efficacy of current treatment with inhaled corticosteroids (ICSs) on small airway inflammation. In Japan between 1997 and 2002, three kinds of ICS drugs were available; dry-powder fluticasone (DP-FP), dry-powder budesonide, meter-dose inhaler of chlorofluorocarbon-beclomethasone (CFC-BDP). The International Commission on Radiological Protection has information on lung deposition, including a good model of deposition efficiency as a function of median particle size, and this model demonstrates that the majority of particles ranging from 2.5 to 6 μm in diameter are deposited in large airways,[3] and those under 2.5 μm are deposited mostly in the alveolar area.[4] In general, the smaller particles can reach the more peripheral airways, but for an optimal therapeutic response to ICSs the inhaled particles should reach both large and small airways. The mass median aerodynamic diameters (MMADs) of CFC-BDP and DP-FP are between 2.5 to 6 μm,[5,6] and total lung deposition rate of these two ICSs are between 10 to 20%.[4,7] Not all asthmatic patients return to normal

[1]Third Department of Internal Medicine, Sapporo Medical University School of Medicine, Hokkaido, Japan.
Correspondence: Hiroshi Tanaka, Associate professor, Third Department of Internal Medicine, Sapporo Medical University School of Medicine, South−1, West−16 Chuo-ku, Sapporo, Hokkaido 060−8543, Japan.
Email: tanakah@sapmed.ac.jp
Received 28 November 2005. Accepted for publication 13 March 2006.

Table 1 Subject characteristics

	Control	Asthma
No. (male/female)	16 (11/5)	16 (12/4)
Age, yr	62.3 ± 2.9	62.2 ± 2.5
Smoking, yes/no	9/7	10/6
Treatment with ICS, yes	0	11
VC, % predicted	96.9 ± 10.7	98.9 ± 6.2
FEV_1/FVC ratio, %	83.6 ± 3.9	66.8 ± 2.9 *
FEV_1, % predicted	96.4 ± 8.0	84.7 ± 7.1
V50, % predicted	80.0 ± 9.3	48.9 ± 8.1 *
V25, % predicted	61.2 ± 3.1	35.5 ± 5.6 *

Data are presented as mean ± SEM/mm^2. ICS = inhaled corticosteroid

* $p < 0.05$ compared with control

Table 2 Characteristics of each asthmatic patient

	Age	Sex	Smoking	Asthma duration	Eo (%)	Serum IgE (IU/ml)	$FEV_{1\%}$ (%)	Treatment
ICS (−) $n = 5$	64	M	yes	8 yrs	4	254	80.0	T, β2
	53	M	no	10 yrs	3	NT	79.2	β2
	70	M	yes	3 yrs	2	34	63.5	T
	74	M	yes	12 yrs	7	102	52.0	T, β2
	68	F	no	7 yrs	2	12	60.4	T, β2
ICS (+) $n = 11$	68	M	no	4 yrs	9	67	70.0	T, β2, BDP 800 μg/day
	76	M	yes	5 yrs	1	57	71.9	T, β2, FP 400 μg/day
	70	M	yes	14 yrs	5	NT	65.7	T, β2, FP 400 μg/day
	50	M	yes	1 yr	2	30	68.0	T, β2, FP 400 μg/day
	50	F	no	12 yrs	4	16	53.0	β2, BDP 400 μg/day
	65	M	yes	7 yrs	5	NT	41.9	T, β2, BDP 400 μg/day
	76	F	no	11 yrs	2	14	84.4	β2, FP 400 μg/day
	55	M	yes	8 yrs	4	312	60.3	β2, FP 800 μg/day
	48	M	yes	20 yrs	12	156	80.7	T, β2, BDP 800 μg/day
	59	F	no	10 yrs	1	54	66.7	T, β2, BDP 800 μg/day
	49	M	yes	5 yrs	2	23	70.3	Salmeterol 100 μg/day, FP 400 μg/day

ICS: inhaled corticosteroid, Eo: peripheral blood eosinophil, NT: not tested

T: slow-releasing theophylline, β2: inhaled β2-agonist

BDP: chlorofluorocarbon-beclomethasone, FP: dry-powder fluticasone

lung function and airway hyperresponsiveness even after intensive treatment with ICS and/or β2-agonists. A possibility exists that since most inhalation formulations have particle sizes too large to reach the small airways, we are not comprehensively treating the airway inflammation and bronchoconstriction. We tested the hypothesis that there was a therapeutic limitation for larger particle ICS in the treatment of small airway disease in asthma. We examined the therapeutic efficacy of CFC-BDP and DP-FP on eosinophilic inflammation in small airways using surgically resected lung specimens from asthmatic patients who had been treated with these ICSs.

METHODS

SUBJECTS

Thirty-two subjects were recruited from the Sapporo Medical University Hospital and Minami Ichi-jo Hospital. They underwent lobectomy or pneumonectomy for small peripheral lung cancer between 1997 and 2002. Asthma was defined according to the criteria of the American Thoracic Society,[8] and the severity defined by the Global Initiative for Asthma.[9] The subject characteristics are shown in Tables 1, 2. Sixteen control subjects who had no history of asthma were selected from the same hospitals' medical file between 1997 and 2002 on the basis of the best match to asth-

Table 3 Inflammatory cells in small airways in control and asthmatic patients

	Control ($n = 16$)		Asthma ($n = 16$)	
Area	inner	outer	inner	outer
Mast cells	84.5 ± 16.1	95.9 ± 15.9	118.5 ± 20.0	111.0 ± 14.1
Macrophages	4.3 ± 1.5	5.9 ± 2.2	3.9 ± 4.1	8.4 ± 1.3
Eosinophils	12.3 ± 12.1	33.1 ± 23.9	94.7 ± 24.5 *	103.9 ± 13.6 *
CD34+ cells	42.4 ± 8.4	33.9 ± 7.1	125.9 ± 9.5 *	65.7 ± 20.1

* $p < 0.05$ compared with control

Mean ± SEM/mm^2

Table 4 Inflammatory cells in inner area of large and small airways in asthmatics patients treated with or without inhaled corticosteroid

		Asthmatic patients		
		ICS (−) $n = 5$	ICS (+) $n = 11$	p-value
Large airways	Mast cells	98.9 ± 17.4	95.3 ± 13.2	0.99
	Macrophages	14.6 ± 6.8	9.1 ± 3.5	0.79
	Eosinophils	173.4 ± 42.9	36.1 ± 19.2	0.013
	CD34+ cells	73.2 ± 10.8	59.1 ± 8.9	0.69
Small airways	Mast cells	135.0 ± 20.0	105.2 ± 18.9	0.29
	Macrophages	7.0 ± 4.1	1.4 ± 1.4	0.34
	Eosinophils	90.0 ± 44.9	100.4 ± 47.5	0.99
	CD34+ cells	122.4 ± 9.5	145.3 ± 39.0	0.93

ICS; inhaled corticosteroid (CFC-BDP, DP-FP)

Mean ± SEM/mm^2

matic patients with respect to age, sex and smoking history. As shown in Table 2, eleven patients with asthma were treated with CFC-BDP, 400–800 μg/day or DP-FP, 400–800 μg/day and the other 5 patients did not receive ICSs. There were no significant differences between the two groups in age, sex, tobacco habit, asthma duration period or $FEV_{1\%}$. This study was approved by the Sapporo Medical University ethics committee, and written informed consent was obtained from all subjects.

IMMUNOHISTOCHEMISTRY

Lung specimens without cancer were fixed with 10% formalin and embedded in paraffin. Sections of 5 μm thickness were deparaffinized with xylene, dehydrated in ethanol, and then heated in a domestic microwave oven (500 watts) for 5 minutes to retrieve antigens. Endogenous peroxidase was blocked by incubation in 1% hydrogen peroxide in methanol for 30 minutes. After washing with phosphate-buffered saline (PBS), they were incubated with 2% normal antibodies for 30 minutes at room temperature. Anti-CD34 monoclonal antibody (Becton Dickinson Immunocytometry Systems, San Jose, USA), anti-CD68 monoclonal antibody (NEO MARKERS, Fremont, USA), anti-tryptase monoclonal antibody (DAKO, Glostrup, Denmark), anti-tryptase monoclonal antibody (DAKO, Glostrup, Denmark), and anti-EG2 monoclonal antibody (Pharmacia&Upjohn, Peapack, USA) were used. They were incubated with biotinylated anti-mouse or rabbit immunoglobulin G (IgG, heavy and light chains H&L) affinity purified antibody for 30 minutes, followed by incubation with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for 30 minutes. They were washed 3 times with PBS for 5 minutes each and immersed in a 0.05% diaminobenzidine tetrahydrochloride solution containing 0.03% hydrogen peroxide for 1–3 minutes followed by counterstaining of the nuclei with hematoxylin solution. Normal mouse Ig was used as a negative control. Two observers who had no knowledge of the subject characteristics counted each inflammatory cell, and each observer examined 5 fields for each patient. The mean coefficient of variation for repeated measurements was 6–10% for each inflammatory cell.

AIRWAY CLASSIFICATION

Small airways were defined as membranous airways with an inner diameter < 2 mm without cartilage. Large airways were defined as airway tissue that contained cartilage with an inner diameter of 2–5 mm.[10,11]

QUANTITATION

Each subject was examined in 5 fields each consisting of medium and small airways. The vascularity and number of cells was counted separately in the inner

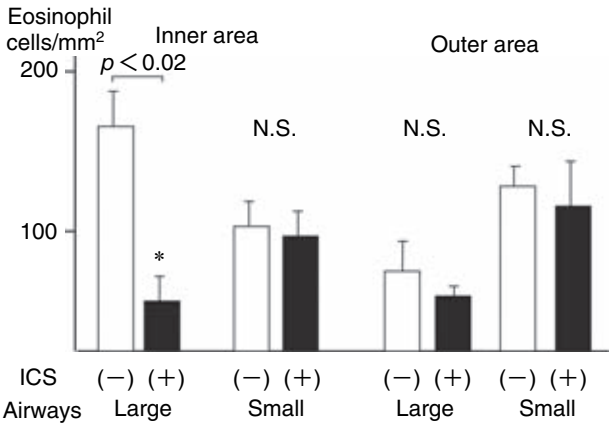

Fig. 1 The number of eosinophils in the inner and outer area of large and small airways in asthmatic patients treated with or without inhaled corticosteroids (ICS). Eosinophil infiltration in the inner area of large airways is significantly suppressed by ICS therapy, but is not inhibited in the inner area of small airways.

airway wall between the basement membrane and the outer border of smooth muscle and in the outer wall between the outer border of the smooth muscle and the parenchyma [2,11] excluding mucous glands, smooth muscle and cartilage. The number of each inflammatory cell per mm^2 was examined using a light microscope with MOTIC 2000 (Shimazu Rikaki, Tokyo Japan) software. Two observers who had no knowledge of the subject characteristics counted the vessels, and each observer examined 5 fields for each patient. The mean coefficient of variation for repeated measurements was 6–10% for each inflammatory cell.

DATA ANALYSIS

The values are presented as mean ± SEM. The Mann-Whitney and χ^2 tests were used for comparison between the two groups. A p value of less than 0.05 was considered statistically significant.

RESULTS

The number of eosinophils in the inner and outer area of small airways was significantly ($p < 0.05$) increased in asthmatics and the number of CD34+cells in the inner area of small airways was significantly ($p < 0.05$) elevated as compared with those of controls (Table 3). The mean number of eosinophils in the outer area of small airways was higher than that in the inner area, but there was no significant difference. On the other hand, in large airways of asthmatics, the number of both eosinophils and CD34+ cell in the inner area were significantly ($p < 0.05$) higher than those in controls (18.3 /mm^2 ± 10.2 *vs* 89.4 ± 19.5, 38.4 /mm^2 ± 7.2 *vs* 98.4 ± 19.5, respectively). No differences were observed in the number of mast cells and macrophages in either small or large airways between asthma patients and controls.

Although the cell density of eosinophils in the inner area of large airways was significantly suppressed ($p < 0.02$) in asthmatic patients treated with CFC-BDP or DP-FP as compared with patients without ICS treatment, there was no such suppression in the inner area of small airways (Table 4, Fig. 1). In the case of mast cells, macrophages, and CD34+ cells, there was no suppression by ICS therapy in either small or large airways (Table 4).

DISCUSSION

Our results demonstrated that eosinophils and CD34+ cells in small airways were significantly increased in asthmatic patients as compared with controls. However mast cells and macrophages were not increased. The CD34 molecule is expressed on pluripotent hematopoietic stem cells as well as on eosinophils, monocytes/macrophages and subsets of lymphocytes, and CD34+ stem cells also express cysteinyl-leukotriene receptor 1 and 2, which differentiate to various inflammatory cells and enhance asthmatic inflammation. Many investigators have reported inflammatory changes in the small airways in subjects with asthma using lung specimens.[2,10,12,13] Saetta *et al.*[13] found evidence of increased mucus plugging, airway smooth muscle thickening, and infiltration of inflammatory cells including eosinophils in the small airways in fatal asthma patients. Hamid *et al.*[2] observed that inflammatory changes were more severe in small airways than in large airways of asthmatic patients who had undergone lung resection for tumor. Kraft *et al.*[14] reported that eosinophilic inflammation in nocturnal asthma was greater in peripheral tissue than in the large bronchi in the early morning hours.

Other studies have shown that, unlike large airways, inflammatory cell infiltration predominates in the outer area of small airways.[15-17] In our study, inflammatory cell density in small airways was not increased in outer areas compared with inner areas. Hamid *et al.*[2] reported a greater number of total eosinophils in the outer layer compared with the inner layer in small airways. It has also been reported that outer area inflammation[16] and destruction of alveolar attachment[18] were more prominent in patients with severe asthma, but in our cases the results showed more prominence in mild and moderate asthma patients. This may be one of the reasons why outer area inflammation was not predominant in the present study.

The density of eosinophils in the inner area of large airways was significantly suppressed ($p < 0.02$), but this suppression of eosinophils was not seen in the inner area of small airways in patients with asthma treated with CFC-BDP or DPI-FP. Hauber *et al.*[19] reported that HFA-flunisolide (MMAR is 1.2 μm) effectively suppressed eosinophilic airway inflamma-

tion in both small and large airways using transbronchial biopsy specimens. It was speculated that the particle size of CFC-BDP and DP-FP prevented them from reaching small airways.

In conclusion, ICSs of CFC-BDP (400–800 μg/day) or DP-FP (400–800 μg/day) markedly reduced eosinophils in large airways, but not in small airways in resected lung specimens of patients with bronchial asthma. Lung deposition rate of ICSs to small airways might be one of the important factors in the treatment of bronchial asthma.

REFERENCES

1. Howarth P. The relevance of and site of airway inflammation in asthma and targeted aerosol delivery. *Int. J. Clin. Pract. Suppl.* 1999;**106**:3-10.
2. Hamid Q, Song Y, Kotsimbos TC *et al.* Inflammation of small airways in asthma. *J. Allergy Clin. Immunol.* 1997; **100**:44-51.
3. Köbrich R, Rudolf G, Stahlhofen W. A mathematical model of mass deposition in man. *Ann. Occup. Hyg.* 1994;**38** (Suppl):15-23.
4. Pritchard JN. The influence of lung deposition on clinical response. *J. Aerosol Med.* 2001;**14**(Suppl 1):S19-S26.
5. Cripps A, Riebe M, Schulze M *et al.* Pharmaceutical transition to non-CFC pressurized metered dose inhalers. *Respir. Med.* 2000;**94**(Suppl):S3-S9.
6. Asmus MJ, Liang J, Coowanitwong I *et al.* Respirable dose of fluticasone from a metered-dose inhaler (MDI) and two dry-powder inhalers (DPI). *Am. J. Respir. Crit. Care Med.* 2001;**163**:A444.
7. Prichard JN, Layzell G, Miller JF. Interpretation of *in vitro* particle size data from dry powder inhalers. In: Dalby R, Byron P, Farr SJ(eds). *Respiratory Drug Delivery VI.* Buffalo: Interpharm Press, 1998;401-404.
8. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am. Rev. Respir. Dis.* 1987; **136**:225-244.
9. National Institutes of Health, National Heart Lung, and Blood Institute. *Global Strategy for Asthma Management and Prevention.* Publication No 02-3659. Betheda: National Institute of Health, 2002.
10. Carroll N, Cooke C, James A. The distribution of eosinophils and lymphocytes in the large and small airways of asthmatics. *Eur. Respir. J.* 1997;**10**:292-300.
11. Hashimoto M, Tanaka H, Abe S. Angiogenesis in inner area of the middle bronchus may correlate with airflow limitation of asthmatics. *Chest* 2005;**127**:965-972.
12. Carroll N, Elliot J, Morton A, James A. The structure of large and small airways in nonfatal and fatal asthma. *Am. Rev. Respir. Dis.* 1993;**147**:405-410.
13. Saetta M, DeStefano A, Rosina C, Thiene G, Fabbri LM. Quantitative structural analysis of peripheral airways and arteries in sudden fatal asthma. *Am. Rev. Respir. Dis.* 1991;**143**:138-143.
14. Kraft M, Djukanovic R, Wilson S, Holgate ST, Martin RJ. Alveolar tissue inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 1996;**154**:1505-1510.
15. Haley KJ, Sunday MF, Wiggs BR *et al.* Inflammatory cell distribution within and along asthmatic airways. *Am. J. Respir. Crit. Care Med.* 1998;**158**:565-572.
16. Carroll N, Carello S, Cooke C, James A. Airway structure and inflammatory cells in fatal attacks of asthma. *Eur. Respir. J.* 1996;**9**:709-715.
17. Carroll N, Mutavdzic S, James AL. Distribution and degranulation of airway mast cell in normal and asthmatic subjects. *Eur. Respir. J.* 2002;**19**:879-885.
18. Mauad T, Silva LFF, Santos MA *et al.* Abnormal alveolar attachments with decreased elastic fiber content in distal lung in fatal asthma. *Am. J. Respir Crit. Care Med.* 2004; **170**:857-862.
19. Hauber HP, Gotfried M, Newman K *et al.* Effect of HFA-flunisolide on peripheral lung inflammation in asthma. *J. Allergy Clin. Immunol.* 2003;**112**:58-63.