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Airway inflammation and basement membrane tenascin in newly diagnosed atopic and nonatopic asthma

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Summary Previous studies have shown both similar and distinct inflammatory changes in atopic and nonatopic asthma. This study was set to investigate the bronchial inflammatory cell infiltrate and subepithelial basement membrane (BM) tenascin deposition in subjects with newly diagnosed asthma and bronchial hyperresponsiveness (BHR). Seventy-nine asthmatic subjects (age 18–60 years) were recruited and 58 were atopic according to skin prick testing. The patients recorded asthma symptoms and peak flow measurements for 14 days. Lung function and BHR were measured by spirometry and histamine challenge. Serum eosinophil cationic protein (ECP) and blood eosinophils were assessed. Fiberoptic bronchoscopy was performed to obtain bronchial biopsies. Serum ECP was higher in the atopic group but eosinophil counts did not differ. There were no differences in inflammatory cells studied (activated eosinophils, T-lymphocytes, mast cells or macrophages) between nonatopic and atopic subjects. BM tenascin layer was significantly thicker in atopic compared with nonatopic subjects (7.6 vs 6.3 μm, $P = 0.007$). The thickness of tenascin correlated with eosinophil, T-lymphocyte, and macrophage counts, as well as with IL-4-positive cell counts and the correlation was seen only in atopic asthmatics. These findings suggest that inflammatory cells may have a regulatory role in tenascin expression in atopic asthma.

Introduction

Asthma is an inflammatory disorder of the airways, which is characterized by reversible airways obstruction causing dyspnea and bronchial hyperresponsiveness (BHR) to specific and nonspecific stimuli. The pathological features in the asthmatic airways are infiltration of inflammatory cells in the mucosa, especially eosinophils and T-lymphocytes, epithelial shedding, goblet cell hyperplasia, and thickening of the basement membrane (BM). These events are seen even in newly diagnosed asthma.[1] Tenascin is a large extracellular matrix (ECM) protein that is expressed in the BM zone during embryonic development, tissue repair, and oncogenesis.[2] Its expression in bronchial BM is elevated in asthma compared with nonasthmatic nonatopic control subjects; and the thickness of tenascin immunoreactivity in atopic asthmatics decreased

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by treatment with budesonide.[3] The role of increased tenascin accumulation in the asthmatic bronchial BM is yet unknown. It may contribute to the ongoing epithelial injury and repair in asthmatic inflammation as it contributes to the wound healing process.[4]

Asthma is divided into two subgroups according to the atopic status. The variants are atopic (atopic) asthma and nonatopic (nonatopic) asthma.[5] The clinical features of these subtypes of asthma are distinct. Patients with nonatopic asthma are more frequently females and their disease has more severe clinical course and the family history of allergy or asthma is often lacking.[6] Atopic asthma is IgE-mediated with raised IgE-antibodies to allergens, whereas in nonatopic asthma IgE-mediated reactions cannot be detected.[7] Nonatopic asthmatics are less hyperresponsive to inhaled adenosine-5′-monophosphate[8] and they have normal concentrations of exhaled nitric oxide opposed to higher than normal concentrations in atopic asthmatics.[9] These findings suggest that bronchial inflammatory changes in atopic and nonatopic asthma are different. However, Humbert and coworkers found similarities in chemokine and cytokine expression in airway mucosa both in nonatopic and atopic asthma.[10] There is preliminary evidence that airway macrophage infiltration and function are not similar in nonatopic and atopic asthma.[6] Amin et al. found that tenascin layer in subepithelial BM was thicker in atopic compared with nonatopic asthmatics showing a structural difference in the bronchial mucosal level in these distinct asthmatic subgroups.[11]

The aim of the present study was to compare inflammatory cell pattern and BM tenascin in bronchial mucosa of atopic and nonatopic subjects with newly diagnosed asthma and BHR.

Methods

Patients

Currently, nonsmoking subjects (nonsmokers or exsmokers >2 years and <10 pack years) aged between 18 and 60 years were recruited to the study. Asthma was diagnosed according to the American Thoracic Society (ATS) guidelines[12] less than 2 years earlier. A 15% reversibility in FEV_1 or peak flow (PEF) or a 20% diurnal variation in PEF were recorded at least 12 months prior entering the study. Subjects were currently symptomatic and all of them were hyperresponsive ($PD15FEV_1$ to histamine <0.4 mg). A the time of selection to the study the FEV_1 was more than 60% of the national predicted value.[13] Before entering the study, the participants received only inhaled short-acting β_2-agonist and they had no inhaled or oral steroids during the last 2 months, no long-acting β_2-agonist or other asthma medication during the last 4 weeks or any anti-histamine for 2 weeks. The patients with serious uncontrolled systemic diseases, current use of beta-blockers, respiratory tract infections, or asthma exacerbation within 4 weeks were excluded. The atopic status was assessed by skin prick testing with a panel of 10 common aeroallergens. The patients who were skin prick test positive for a seasonal allergen were not allowed to participate in the study during the season of their allergy. All patients gave informed, written consent before commencing the study. The study was approved by the local ethics committee.

Patients monitored their PEF, symptoms, and rescue medication need (salbutamol pMDI 0.1 mg/dose: Ventoline®) for a period of 2 weeks before the bronchoscopy. The daytime symptoms were recorded on the diary: from 0 as no symptoms to 5 as incapacitating symptoms. The nighttime symptoms were recorded similarly, but from 0 to 4. PEF was measured with a mini-Wright peak flow meter (Clement-Clarke, Harlow, UK) in the morning and evening. The patient recorded the best of three measurements. The inhalation of rescue bronchodilator was avoided 4 h before the PEF measurement.

FEV_1 and forced vital capacity (FVC) were measured with a Vitalograph TM spirometer (Fleisch, Bucks, UK) before entering the study to evaluate the entry criteria and in the morning on the day of bronchoscopy following the ATS standard.[12] FEV_1 and FVC assessed again 15 min later after inhalation of 0.2 mg of salbutamol to test for reversibility. Histamine challenge was performed 2–4 weeks before bronchoscopy with the use of an inhalation-synchronized, dosimetric nebulizer (Spira Elektro 2, Respiratory Care Center, Hämeenlinna, Finland).[14]

Blood samples were collected before the bronchoscopy to determine blood eosinophils and eosinophil cationic protein (ECP) in the serum. The number of blood eosinophils was counted in Bürkes chamber after staining with floxine. Serum ECP was measured by an immunofluorometric method on a fully automated immunoanalyzer UniCap 100 (Pharmacia & Upjohn, Uppsala, Sweden). The normal range for serum ECP was considered from 2 to 16 μg/l.

Fiberoptic bronchoscopy was performed according to the international standard.[15] Premedication included oral oxazepam 30 mg, intramuscular

atropine 0.5 mg, and inhaled salbutamol 0.2 mg. Lignocaine was used as local anesthetic up to a maximum dose of 500 mg. The bronchoscope (Olympus XT20, Tokyo, Japan) was introduced orally into the right main bronchus. The biopsy specimens were taken using cupped forceps (FB-36C E, Olympus, Japan) from unattached areas of bronchial mucosa: inside the right upper lobe, at the opening of the right middle lobe, and from one of the basal segment bronchi of the right lower lobe.

Sample processing and immunohistochemistry

The biopsy specimens were immediately snap frozen in liquid nitrogen, stored at -70°C and processed as previously described.[3]

Mast cells, macrophages, T-lymphocytes, as well as their subsets, and neutrophils were identified with the following mouse monoclonal antibodies (Mab's) mast cell tryptase clon AA1 (1:500), BerMac3 (1:50), anti-CD3 (1:1000), anti-CD4 (1:100), anti-CD8 (1:1000) and anti-neutrophil elastase clone NP57 (1:2000, ELA) from Dako A/S (Glostrup, Denmark), respectively. To identify IL-4-positive cells mouse monoclonal anti-human IL-4 (1:50) from Gemzyme Diagnostics (Cambridge, MA, USA). The cells were visualized with the alkaline phospatase anti-alkaline phosphatase technique in accordance with the manufacturer's instructions (Dako A/S, Glostrup, Denmark). To detect eosinophils, the frozen sections were fixed with 4% paraformaldehyde in phosphate buffer at $+20^{\circ}$C for 20 min, and then exposed 30 min to normal rabbit serum 1:10 following the incubation with the primary Mab EG2 (1:50, Kabi Pharmacia Diagnostics AB, Uppsala, Sweden) over night at $+4^{\circ}$C and processed for the avidin–biotin–peroxidase method in accordance with the manufacturer's instructions. The sections were examined and photographed under a Leitz Dialux 22 EB light microscope. Photographic slides were projected onto a calibrated digitizing tablet (Kurta IS/THREE; Kurta Corp., Phoenix, AZ, USA). The density of inflammatory cells in the entire sections was computed with the Autocad program 10.1 (Autodesk Inc., Sausalito, CA, USA) as described previously.[3] Tenascin was detected applying immunofluorescence technique with mouse Mab 100EB2 (Locus Genex, Helsinki, Finland). Areas containing cross-sections of the BM were photographed using Leitz Aristoplan microscope, equipped with appropriate filters, and the thickness of the immunoreactivity was measured semi-automatically using a computerized image-analysis program as described earlier.[3] Negative controls were provided by omitting the primary Mab or replacing it with an irrelevant one.

Statistical analysis

Comparisons between atopic and nonatopic subject groups were performed with Student's *t*-test or Mann–Whitney *U*-test when appropriate. Correlation coefficients (ρ) were calculated with Spearman's rank method. Two-tailed *P* values <0.05 were considered significant.

Results

A total of 79 patients were included to the study. Twenty-one of them were skin prick negative and considered as nonatopic asthmatic. The patients with nonatopic asthma were significantly older but no other significant differences were seen in the demographic or lung function data (Table 1). Asthma duration was similar in both groups. The median daytime symptom score was higher in nonatopic asthmatic subjects ($P = 0.03$), but the use of rescue medication was not increased compared with atopic subjects. Serum ECP was higher in atopic subjects (Table 1). No difference was seen in blood eosinophil counts between nonatopic and atopic asthma.

Bronchoscopy and bronchial biopsies were well tolerated by all patients. Assessable bronchial biopsies were obtained from 63 to 74 subjects for each immunohistochemical staining. In five subjects the biopsy samples were not of appropriate quality for the analysis, and in nine subjects, the assessable material was sparse and not sufficient for all analyses intended. In the entire study group the subepithelial BM tenascin layer thickness correlated positively with the densities of activated eosinophils, CD3+ T-lymphocytes, CD4+ T-lymphocytes, CD8+ T-lymphocytes and, macrophage, but not with mast cells or neutrophils (Table 3). Neither blood eosinophils nor serum ECP correlated significantly with FEV_1, $PD15FEV_1$, inflammatory cell counts, or tenascin layer thickness.

There was no significant difference between nonatopic and atopic subjects for the inflammatory cell counts (Table 2). The thickness of subepithelial BM tenascin layer was significantly greater in atopic subjects (7.6 vs 6.3 μm, $P = 0.007$) (Fig. 1). In atopic subjects, the thickness of tenascin layer correlated significantly correlations with most of the inflammatory cell types studied with exception for mast cells, whereas in nonatopic subjects, a positive correlation was seen only in CD8+ cells

Table 1 Clinical characteristics of the study subjects.

	Nonatopic asthma ($n = 21$)	Atopic asthma ($n = 58$)	All ($n = 79$)
Age (years)†	44 (22–58)*	33 (18–61)	36 (18–61)
Male/female	9/12	24/34	33/46
Asthma duration (years)†	0.25 (0.02–1.67)	0.36 (0–2)	0.33 (0–2)
FEV_1 (L)†	2.90 (1.83–4.38)	3.15 (1.87–4.43)	3.08 (1.83–4.43)
Post-$BDFEV_1$ (L)†	3.17 (1.95–4.61)	3.50 (2.25–4.95)	3.41 (1.95–4.95)
FEV_1 reversibility (%)†	9.9 (3.9–24.8)	11.6 (−1.7–56.4)	11.2 (−1.7–56.4)
$PD15FEV_1$ (mg of histamine)†	0.15 (0.002–0.37)	0.13 (0.001–0.34)	0.13 (0.001–0.37)
Mean morning PEF (L/min)†	449 (310–630)	455 (232–661)	453 (231–661)
Day symptom score‡	1.42 (0–3)*	0.97 (0–3)	1.1 (0–3)
Night symptom score‡	0.7 (0–2)	0.45 (0–2)	0.5 (0–2)
Rescue medication (puffs/daytime)‡	1.32 (0–3.36)	1.34 (0–9.3)	1.2 (0.04–6.0)
S-ECP (μg/l)†	16.8 (2.1–69.4)†	26.1 (2.8–120)	23.6 (2.1–120)
B-EOS (10^9/L)†	0.32 (0.1–1.26)	0.38 (0.1–1.67)	0.36 (0.1–1.67)

*$P < 0.05$.†Mean values (ranges) are presented.‡Median values (ranges) are presented.

(Table 3). EG2+ cells correlated negatively with $PD15FEV_1$ in atopic subjects ($\rho = -0.37$, $P = 0.007$) but not in nonatopic subjects ($\rho = 0.01$, $P = 0.95$).

Discussion

This study reports features of inflammatory changes in bronchial mucosa in a large sample of subjects with newly diagnosed asthma and with BHR. Most of the patients had not received any anti-inflammatory treatment, and all had been only on rescue β_2-agonists for at least 4 weeks before entering the study. We observed a correlation of subepithelial tenascin layer thickness and inflammatory cell infiltration in airway mucosa. This correlation appeared to be more evident in the atopic asthmatic subjects. The inflammatory cell densities in the bronchial mucosa did not distinguish nonatopic asthmatics from atopic asthmatics, but the subepithelial BM tenascin deposition was significantly higher in atopic asthmatics.

ECM provides a physical framework for cells during tissue morphogenesis, homeastasis, and remodeling. Tenascin is an oligomeric glycoprotein that is an important component of ECM. Tn expression is restricted normally to embryonic development. In adult tissues it is expressed only in pathological states or normal reparatory processes as in wound healing and inflammation.[16] Its re-expression in the bronchial subepithelial BM of asthmatic patients reflects ongoing injury–repair process. The mechanisms behind the upregulation of tenascin expression in the airways is mostly unknown. Tenascin expression was induced by TGF-β and TNF-α in cultured bronchial epithelial cells.[17,18] Elevated TGF-β levels have been shown in the bronchoalveolar lavage fluid in asthmatic patients.[19] In the present study, tenascin layer thickness correlated with inflammatory cell densities in the airway mucosa, suggesting that tenascin expression in asthma is associated closely with the extent of the inflammatory process. We observed the strongest correlation in EG2+ cells, but also T-lymphocytes and macrophages correlated significantly with tenascin thickness. Eosinophils are the main effector cells in asthma. By releasing cytotoxic protein such as ECP, major basic protein, eosinophil protein, and eosinophil peroxidase, eosinophils orchestrate the desquamation and destruction of bronchial epithelium.[20] This injury caused by eosinophils may induce the expression of tenascin in the bronchial mucosa. Amin et al.[11] observed that atopic asthmatics had a thicker BM tenascin than nonatopic asthmatic or healthy controls. They also found a negative relationship between epithelial integrity and airway eosinophils in atopic asthmatics. These findings along with our own results of tenascins' relationship with

Table 2 Bronchial biopsy results.

Positive cell or BM component	Nonatopic asthma ($n = 16 - 21$)	Atopic asthma ($n = 47 - 54$)	All ($n = 63 - 74$)
AA1*	86 ± 14 (58, 115)	99 ± 12 (75, 123)	96 ± 9 (77, 115)
BERMAC	255 ± 43 (166, 345)	228 ± 23 (183, 274)	236 ± 20 (196, 276)
CD3*	602 ± 123 (344, 859)	501 ± 48 (404, 598)	529 ± 48 (432, 627)
CD4*	262 ± 57 (142, 383)	237 ± 25 (186, 288)	244 ± 23 (197, 290)
CD8*	187 ± 32 (120, 255)	246 ± 26 (195, 298)	231 ± 21 (189, 273)
EG2*	114 ± 23 (63, 164)	99 ± 12 (75, 123)	103 ± 11 (82, 125)
ELA*	37 ± 10 (17, 57)	40 ± 5 (29, 505)	39 ± 5 (29, 48)
IL4	73 ± 19 (34, 111)	72 ± 9 (54, 90)	72 ± 8 (56, 89)
Tenascin†	6.3 ± 1.2‡ (5.7, 6.9)	7.6 ± 0.3 (6.9, 8.2)	7.2 ± 0.3 (6.7, 7.7)

Abbreviations: AA1 = tryptase positive mast cells, BerMac = macrophages, CD3 = T-lymphocytse, CD4 = helper T-lymphocytse, CD8 = suppressor T-lymphocytes, EG2 = activated eosinophils, ELA = neuthophils, IL4 = interleukin-4-positive cells. Results are given as mean with standard error of mean (95% confidence interval), and expressed as *cells/mm^{-2} or as †μm of immunoreactive thickness of BM.
‡$P = 0.007$ when compared with atopic asthma.

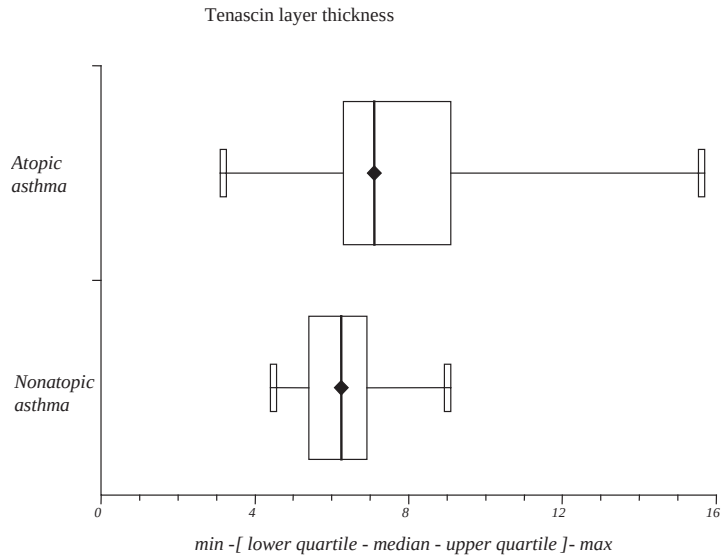

Figure 1 Thickness of tenascin immunoreactive band (μm) in subepithelial BM zone in atopic and nonatopic asthmatics. Horizontal bar indicates median value.

Table 3 Correlations between inflammatory cell densities and tenascin layer thickness.

	Nonatopic asthma (95% CI)	Atopic asthma (95% CI)	All (95% CI)
EG2	0.15 (−0.31, 0.56)	0.38† (0.12, 0.59)	0.32† (0.09, 0.51)
BERMAC	0.25 (−0.22, 0.62)	0.34* (0.07, 0.56)	0.32† (0.09, 0.61)
CD3	0.10 (−0.31, 0.56)	0.32* (−0.01, 0.55)	0.27* (0.04, 0.48)
CD4	0.45 (−0.54, 0.32)	0.36* (0.08, 0.58)	0.34† (0.10, 0.54)
CD8	0.55* (0.07, 0.82)	0.34* (0.06, 0.57)	0.42‡ (0.19, 0.16)
AA1	−0.35 (−0.41, 0.47)	0.12 (−0.41, 0.47)	0.002 (−0.21, 0.25)
ELA	0.04 (−0.41, 0.47)	−0.02 (−0.29, 0.26)	−0.005 (−0.24, 0.23)
IL4	0.35 (−0.12, 0.68)	0.29* (0.002, 0.52)	0.29* (0.04, 0.49)

See Table 2 for abbreviations. Values are Spearman's correlation coefficient (ρ) numbers (95% confidence interval for ρ).
*$P<0.05$.†$P<0.01$.‡$P<0.001$.

eosinophils and other inflammatory cells, let us hypothesize that tenascin expression is associated with epithelial damage caused by inflammatory cells. In asthma epithelial damage and repair is a complex process where a variety of cytokines and growth factors are involved in epithelial–mesenchymal interaction.[21] However, in this study epithelial integrity was not investigated, and further research is needed to study the role of tenascin in epithelial damage. Tenascin has both adhesive and de-adhesive properties,[16] and thus it can be involved in the epithelial shedding and restoration. Our previous study demonstrated tenascin expression in bronchial mucosa in asthma, but it did not show correlation between inflammatory cells and tenascin.[3] However, the patients in the present study were different as having considerably shorter duration of the disease compared with seasonal and chronic asthma patients studied previously.

The immunopathological differences between nonatopic and atopic asthma are still unclear. Our finding of similar peripheral blood eosinophil counts in both types of asthma is in accordance with a previous report.[22] In this study, serum ECP exceeded the normal range in both nonatopic and atopic asthma, but it was higher in atopic subjects suggesting differences in the eosinophil function in the two asthma phenotypes. In the present study, serum ECP level was not associated with lung function or BHR as has been shown earlier.[23] The finding of thicker subepithelial BM tenascin layer in atopic asthmatics reinforces recent observations by Amin et al.[11] Correlation between BM tenascin and inflammatory cells were more pronounced in atopic asthma, which may indicate more uniform structural changes in atopic than in nonatopic asthma. Both differences and similarities have been reported considering the inflammatory cell and cytokine patterns in these asthma types.[10,11,24] In the present study we found no differences in inflammatory cell counts or IL-4-positive cells in the bronchial mucosal level. To our knowledge this is the largest bronchial biopsy study comparing immunopathologic changes in nonatopic and atopic asthmatics groups and therefore provides more evidence supporting the theory of shared immunopathologic mechanisms nonatopic and atopic asthma.

In conclusion, we have demonstrated a similar inflammatory cell pattern in nonatopic and atopic newly diagnosed asthma, but we have also shown that the subepithelial BM tenascin layer is thicker in atopic asthma. We have found shared and distinct features in the bronchial mucosal level in these two asthma phenotypes. We also have

demonstrated a correlation between tenascin BM layer thickness and airway inflammation. Our observation may in part explain the induction of this ECM protein not normally expressed in adult tissues. However, further research is necessary to study the role and regulatory factors of tenascin in asthmatic inflammation.

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