

Influence of interleukin-13 on β -catenin levels in eosinophilic chronic rhinosinusitis cell culture

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Received August 27, 2007; Accepted November 30, 2007

Abstract. Chronic rhinosinusitis (CRS) is one of the most common chronic diseases. The etiology and classification of CRS, with and without nasal polyps, remain unclear. Eosinophils and their products are important in the pathophysiology of allergic diseases and in host immunity to certain organisms. Interleukin 13 (IL-13) plays a pivotal role in eosinophilic inflammation. The migration of epithelial cells requires permanent re-establishment of the intercellular connection. Intercellular connections are maintained by the modulation of adherens junctions consisting of an E-cadherin/ β -catenin complex. In our study we examined the eosinophilic and non-eosinophilic paranasal mucosa obtained from two patients undergoing functional endoscopic sinus surgery. Cell cultures were incubated with human recombinant IL-13 for up to 72 h and β -catenin concentration was determined with ELISA techniques. Furthermore, immunostaining for β -catenin was used for the semi-quantitative description of specimens. We were able to ascertain a significant increase in β -catenin expression in the eosinophilic paranasal cell culture after IL-13 administration compared to the non-eosinophilic culture. Immunostaining for β -catenin was restricted to the membrane of the cells. Concerning the increased mural expression of β -catenin, we presume that a fibrotic reaction similar to asthma and chronic obstructive pulmonary disease occurs in patients suffering from CRS. Furthermore, β -catenin overexpression might be responsible for mucosal thickening and IL-13 seems to be an important marker in eosinophilic CRS.

Introduction

Chronic rhinosinusitis (CRS) is one of the most common chronic inflammatory diseases, and has been defined as a disease of the nasal and paranasal sinus mucosa persisting for more than 3 months, with mucosal changes ranging from inflammatory thickening to nasal polyps (1). Accumulations of activated eosinophils within tissues are the hallmark of this condition; however, the etiology and pathophysiology of CRS are poorly understood (2). Activated eosinophils contribute to nasal polyp pathology by virtue of basic protein deposition, epithelial disruption and basement membrane denudation, and through production of inflammatory mediators and cytokines (3,4). CRS is frequently observed in patients with asthma, and the two diseases have a number of common pathological features: the activation of T-helper type 2 (Th2)-like lymphocytes and eosinophils secreting interleukin (IL)-3, IL-5, IL-13, eotaxin and granulocyte-macrophage colony stimulating factor (GM-SCF). In consequence some authors describe CRS as the 'asthma of the upper airways' (2,5). In the establishment of nonallergic CRS, IL-8, which is generated by neutrophils and mucosal epithelia, has been reported to play a pivotal role (6). Tokushige *et al* proposed that IL-1 production by neutrophils induced the expression of the intercellular adhesion molecule (ICAM-1) on endothelial cells, leading to neutrophil infiltration in CRS (7). It has been demonstrated that the peripheral blood mononuclear cells (PBMCs) of patients suffering from CRS produced large amounts of IL-13 when exposed *in vitro* to *Alternaria* species (8). No similar findings were noted with PBMCs from non-diseased individuals. In CRS, patients showed increases in both Th1 (IFN- γ) and Th2 (IL-5, IL-13) immune responses directed toward *Alternaria* but no shift in the Th1/Th2 balance (8). In contrast, an increased Th2 and decreased Th1 imbalance has been reported to play a key role in the pathogenesis of asthma (9). In CRS, collaborative effects of Th1 and Th2 immune responses to fungi may be responsible for the inflammation and remodeling of sinonasal airways.

The adherens junctions of keratinocytes are composed of cadherin, β -catenin, α -catenin and p120 (10-14). These substructures guarantee the integrity and maintenance of organized

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Key words: interleukin-13, β -catenin, chronic rhinosinusitis, culture, functional endoscopic sinus surgery

tissue. β -catenin was initially discovered as a 96-kDa cadherin-associated protein mediating the anchoring of cadherins to actin by binding α -catenin and its key component of the Wnt signalling pathway (15). Cell-cell adhesion is assumed to be controlled by tyrosine phosphorylation of the adherens junction and desmosome components (15-17). The level of cadherin expression seems to influence the strength and stability of adhesion (18). The dysfunction of β -catenin/E-cadherin complex is suggested to mediate as a factor for the invasiveness and destructive growth of tumour cells into adjacent tissue because of a resulting loss of regular cell adhesion (18). Carayol *et al* induced a decrease in β -catenin expression in human nasal epithelial tissue by TNF- α (19). This decrease was inhibited by the addition of dexamethasone (19).

The cytochemokines (CC) thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) are ligands for the CC chemokine receptor 4 (CCR4), which is expressed selectively on Th2 cells. Ligation of TARC/MDC and CCR4 plays an important role in the migration of Th2 cells into inflamed tissues (20,21). Immunohistologic staining has revealed that keratinocytes express TARC and MDC in the lesional, but not in the nonlesional, skin of atopic dermatitis *in vivo* (22-24). In a nontumorigenic human HaCaT keratinocyte cell line the synthesis and secretion of TARC has been observed after stimulation with TNF- α (25). In HaCaT keratinocyte cell lines, IL-4/IL-13 stimulation reduced staining for E-cadherin and catenins (26). Thus, IL-13 may enhance the internalization of the adherens junction complex, which leads to a down-regulation of TARC/MDC production.

We hypothesized that IL-13 administration might increase β -catenin expression in CRS cell culture. This study was designed to analyse the expression of β -catenin contributing to cell-cell adhesion in CRS tissue. We describe the expression of β -catenin in CRS in comparison with non-eosinophilic paranasal sinus mucosa.

Materials and methods

Tissue collection and culture of human chronic rhinosinusitis epithelial cells. All CRS cells were obtained from 4 patients suffering from CRS and undergoing functional endoscopic sinus surgery at the Department of Otorhinolaryngology at the University of Mannheim, Germany in 2006. Prior to surgery, written consent was obtained from all patients for the use of tissue samples of the resected paranasal mucosa and turbinates. After surgery the tissue samples were examined by a pathologist and diagnosed as eosinophilic and non-eosinophilic CRS depending on the amount of eosinophilic granulocytes in the samples. We set up an epithelial culture for each sample of paranasal sinus mucosa and inferior nasal turbinate. After removal of connective tissue, the tissue specimens were cut into small pieces and incubated in trypsin solution (0.25% trypsin in phosphate-buffered saline, PBS) overnight at 4°C. For the primary culture of epithelial cells, the suspension was added onto mitomycin-treated (23.9 μ M) human fibroblast monolayers and cultured in FAD2 medium (Dulbecco's modified Eagle's medium and Ham's F12 in a 3:1 ratio supplemented with fetal calf serum, adenine,

insulin, triiodothyronin hydrocortisone, epidermal growth factor, cholera toxin and penicillin/streptomycin) at 37°C in a 10% CO₂ atmosphere. On reaching subconfluency, the feeder layer was removed by incubation with 0.02% ethylenediamine tetraacetic acid (EDTA) in PBS for 4 min at 37°C and the sinus epithelial cells were further cultured in keratinocyte growth medium (KGM, Clonetics, San Diego, CA, USA) without serum. Cells were passaged by trypsinization (0.1% trypsin and 0.02% EDTA dissolved in PBS, 5 min, 37°C).

β -catenin ELISA principle. We used the Quantikine β -catenin Immunoassay from R&D Systems (Minneapolis, MN, USA) to determine the exact concentration of β -catenin in the cell culture supernatants. The assay employed the quantitative sandwich enzyme immunoassay technique (ELISA). A monoclonal antibody specific for β -catenin was pre-coated onto a microplate. Standards and samples were pipetted into wells and any β -catenin present was bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for β -catenin was added to the wells. Following a wash to remove the unbound antibody enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of β -catenin bound in the initial step. The color development was stopped and the intensity of the color was measured. The cells were grown in 96-well plates (part 890218) with 12 strips of 8 wells coated with mouse antibody against β -catenin. After 8, 24, 48 and 72 h of incubation with 1 ng/ml human recombinant IL-13 (catalog no. 213-IL) from R&D Systems, the expression of the β -catenin protein in the supernatants of the IL-13 and untreated culture cell lines was analysed. Concurrently, cultured CRS cells were incubated with different levels of IL-13 (1-6 ng/ml) for up to 72 h.

Immunohistochemistry. Immunohistochemical analysis was performed using a monoclonal mouse anti-human antibody directed against β -catenin (C19220, Transduction Laboratories, Lexington, KY, USA). Immunostaining was performed using the alkaline phosphatase-anti-alkaline phosphatase method (APAAP). The sections received a microwave pre-treatment, which required boiling for 15 min at 600 W using citrate buffer for β -catenin. The following steps were performed using an automated staining system, Dako TechMate 500 (Dako, Hamburg, Germany). The sections were incubated with the primary antibody solution for 25 min at room temperature, using a working dilution of the antibody 1:300 for β -catenin. Slides were rinsed once in the buffer (Puffer Kit, Dako). Immunoreaction was demonstrated with the Dako ChemMate Detection Kit (APAAP, mouse, code no. K 5000), according to the specifications of the manufacturer. The sections were incubated with the chromogen alkaline-phosphatase substrate (Neufuchsin, Dako) for 20 min at room temperature. Finally, the sections were counterstained by Mayer's hematoxylin for 3 min, dehydrated in graded ethanol, and coverslipped. Negative controls used all reagents except the primary antibody.

Analysis of β -catenin-immunostaining. The rates of expression were analysed semi-quantitatively. The number of positively marked epithelial cells was graded 0 (no positive



Incubation time (h)	IL-13 (ng/ml)			
	0	1	3	6
	β-catenin: median/standard division (ng/ml)			
8	12.37/1.856	18.235/7.797	16.358/2.661	16.480/1.841
24	12.75/2.5905	19.083/7.983	17.063/2.788	15.878/4.810
48	12.43/1.873	19.59/7.832	18.295/2.490	17.253/4.666
72	10.885/1.050	20.003/8.158	19.363/3.168	21.883/5.005
	β-catenin percentage of total protein (%)			
8	0.010	0.007	0.004	0.004
24	0.003	0.028	0.008	0.010
48	0.003	0.008	0.009	0.008
72	0.003	0.010	0.010	0.010

cells), 1 (<20% positive cells), 2 (20-50% positive cells) and 3 (>50% positive cells). The intensity was noted as I (faint) or II (strong). The combination of these immunohistochemical reaction patterns resulted in 7 possible scores: 0, 1/I, 2/I, 2/II, 3/I, and 3/II. The reaction scores 0 to 2/I were classified as negative or low expression and 2/II to 3/II as high expression of β-catenin.

Statistical analysis. We used the t-test in order to evaluate the p-value ($p < 0.05$) of differences between cultures treated with IL-13 and the control groups. The influence of the various IL-13 concentrations and incubation time was analysed using the GLM procedure.

Results

Incubation up to 72 h with IL-13. Regarding the total protein concentration, after 3 days of incubation with IL-13, the levels of β-catenin increased. After 8 h of incubation with 1 ng/ml IL-13 in epithelial cell culture of eosinophilic paranasal mucosa, the average concentration of β-catenin was 18.235 ng/ml. The levels of β-catenin after 24 h in the same eosinophilic cultures were 19.0825 ng/ml. Increase of β-catenin concentration was ~5%. After 48 h, the further increase was 3% (19.59 ng/ml). The total concentration of β-catenin reached a maximum after 72 h of incubation, 20.0225 ng/ml (increase of 2%), whereas in the control the concentration of β-catenin decreased ~7%. The percentage of β-catenin from the total protein concentration increased from 0.008 to 0.01% (Table I).

At 3 ng/ml IL-13, the average β-catenin concentration in eosinophilic culture was 16.3575 ng/ml after 8 h of incubation and showed a total increase of β-catenin of 19.3625 ng/ml after 72 h of incubation. In the control, the β-catenin concentration decreased from 11.635 ng/ml after 8 h of incubation to 11.4825 ng/ml after 72 h of incubation. The percentage of β-catenin from the total protein concentration dropped from 0.008 to 0.003% in the control (Table I).

At 6 ng/ml IL-13, the average β-catenin concentration in eosinophilic culture was 16.48 ng/ml after 8 h of incubation and reached a maximum of 21.8825 ng/ml β-catenin after 72 h of incubation. However the percentage of β-catenin from total protein showed an identical increase of 0.008 to 0.003% for the incubation with 1 and 3 ng/ml IL-13. The GLM procedure showed no significant influence of the concentration of IL-13 on the total β-catenin concentration ($p = 0.5124$) (Fig. 1A-D).

However, the incubation with IL-13 showed a significant difference in the β-catenin concentration of the eosinophilic paranasal tissue culture compared to the control ($p < 0.0001$).

In non-eosinophilic paranasal tissue culture the β-catenin concentration was not significantly different for incubation with 1 to 6 ng/ml IL-13 ($p = 0.7016$).

Comparing total protein content and the fraction of β-catenin at different points in time in the eosinophilic cultures of inferior turbinates, the concentration of β-catenin increased in cultures of 1 ng/ml (21.8875 ng/ml, 0.007%), 3 ng/ml (20.1975 ng/ml, 0.006%) and 6 ng/ml (24.2675 ng/ml, 0.008%) respectively. The expression of β-catenin was significantly different in eosinophilic turbinate cultures compared with the control culture ($p < 0.0001$). The non-eosinophilic turbinate culture also revealed a significant difference in β-catenin concentration in comparison to the control group ($p = 0.0401$).

Further statistical analysis was performed in order to evaluate the influence of IL-13 concentration, eosinophils and hours of incubation. The analysis of variance following the GLM procedure revealed a significant influence of eosinophils ($p < 0.0001$) and incubation time ($p < 0.0001$) in eosinophilic paranasal tissue. In terms of IL-13, the concentration of IL-13 failed to show significant influence on the β-catenin concentration in these cultures ($p = 0.4373$) (Table I).

Immunohistochemistry. The basal layer showed a high reactivity for β-catenin. The immunostaining was restricted to the membrane of the cells. Comparing the immunoreactivity

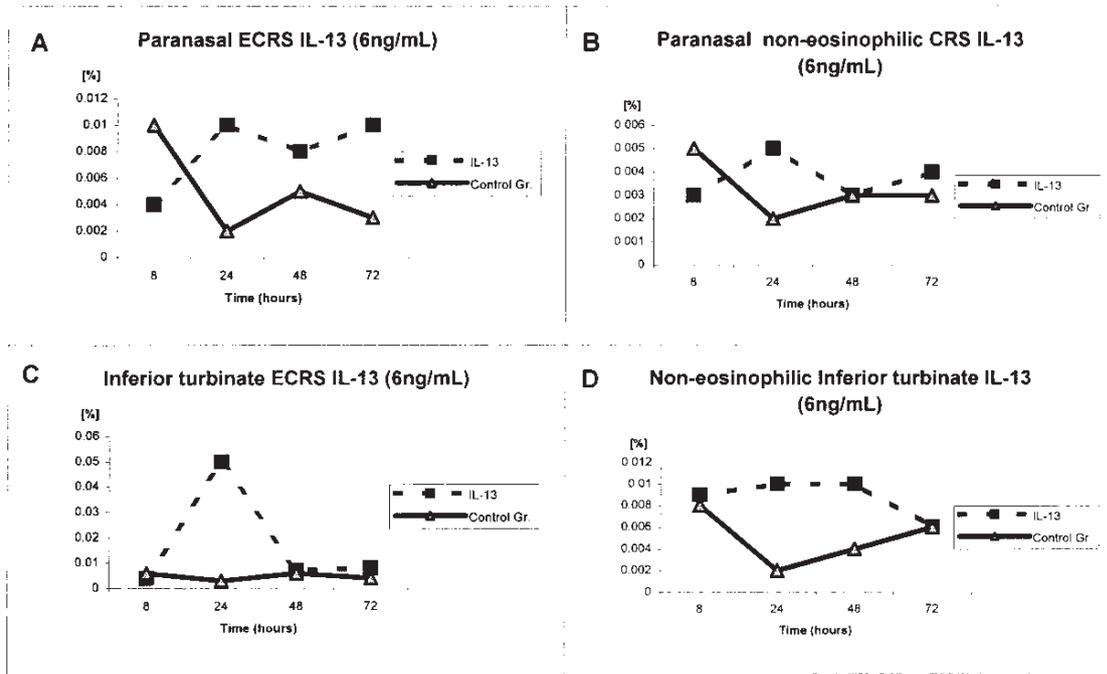


Figure 1. β -catenin-percentage of total protein. Results of the ELISA (incubation with 6 ng/ml for 8, 24, 48 and 72 h). (A) Culture of paranasal eosinophilic CRS. (B) Culture of non-eosinophilic paranasal CRS. (C) Culture of inferior turbinate eosinophilic CRS. (D) Culture of non-eosinophilic inferior turbinate CRS.

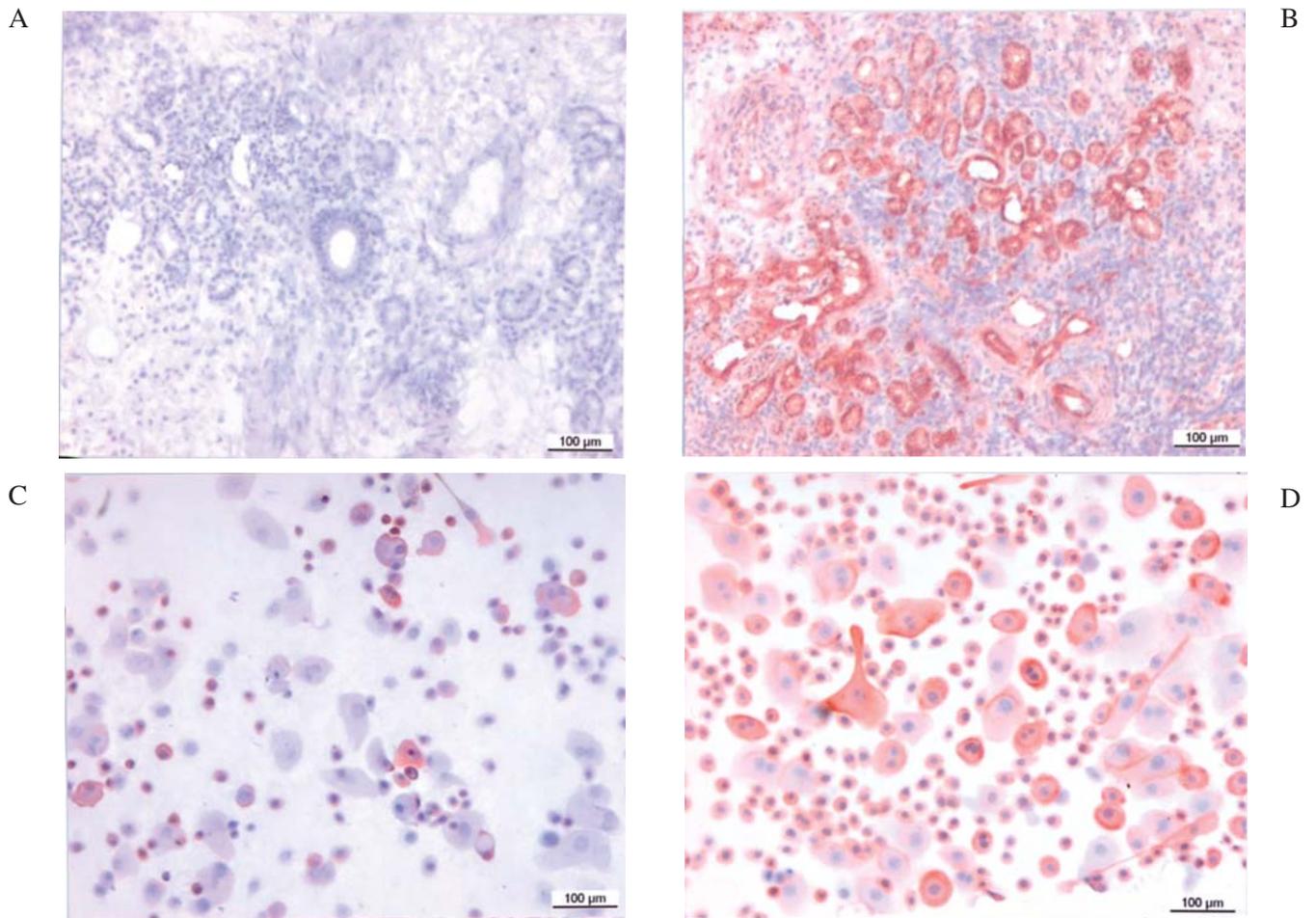


Figure 2. (A) Immunohistochemical illustration of the inferior turbinate (hematoxylin and eosin). (B) Immunohistochemical activity against β -catenin in the inferior turbinate. (C) Immunohistochemical analysis of β -catenin activity in a culture of non-eosinophilic CRS without IL-13 application and (D) in culture of paranasal eosinophilic CRS after 24 h of incubation with 6 ng/ml IL-13.

SPANDIDOS PUBLICATIONS Immunohistochemical score (IS). Grading of eosinophilic and non-eosinophilic CRS.

Tissue	IS ^a	Immunoreactivity of β -catenin (n=4)	
		IL-13 (6 ng/ml)	Control
ECRS	0-2/I	1	x
Sinus mucosa	2/II-3/II	3	
Incubation time: 1 day			
NECRS	0-2/I	3	x
Sinus mucosa	2/II-3/II	1	
Incubation time: 1 day			
ECRS	0-2/I	0	x
Sinus mucosa	2/II-3/II	4	
Incubation time: 3 days			
NECRS	0-2/I	2	x
Sinus mucosa	2/II-3/II	2	
Incubation time: 3 days			
ECRS	0-2/I	2	x
Turbinates	2/II-3/II	2	
Incubation time: 1 day			
NECRS	0-2/I	3	x
Turbinates	2/II-3/II	1	
Incubation time: 1 day			
ECRS	0-2/I	2	
Turbinates	2/II-3/II	2	x
Incubation time: 3 days			
NECRS	0-2/I	4	x
Turbinates	2/II-3/II	0	
Incubation time: 3 days			

^a0-2/I, negative to low expression of β -catenin; 2/II-3/II, strong expression of β -catenin. x, control culture without IL-13 incubation; ECRS, eosinophilic chronic rhinosinusitis; NECRS, non-eosinophilic chronic rhinosinusitis.

of the basal layer of non-eosinophilic paranasal mucosa and eosinophilic paranasal mucosa in CRS there was no difference in β -catenin expression. However, the eosinophilic paranasal mucosa samples showed a high mural expression of β -catenin. The results are presented in Fig. 2A-D and Table II.

Discussion

β -catenin was identified as a crucial link in the cadherin-catenin complex (10). Transducing via the intracellular component α -catenin by arranging the binding to cytosolic filaments and the extracellular component E-cadherin, β -catenin affects cell adhesion in epithelial tissue (15). It has been reported that the migration of inflammatory cells reduces the intensity of

immunofluorescence of E-cadherin in cultured human nasal epithelial cells (27). However, the authors reported that the degree of decrease did not differ in eosinophils compared to the decrease induced by neutrophils. Furthermore, the degranulation extracts from stimulated eosinophils were not able to induce a change in E-cadherin expression by themselves. Nevertheless a loss of epithelial E-cadherin was observed after infiltration through eosinophils (28). The effect of cell transmigration on the expression of cadherins and catenins in tissue-resident cells was reported in the relation between polymorphonuclear leucocytes and vascular endothelial cells (29). E-cadherin and β -catenin are potent adhesion molecules and blockage of these adhesion molecules induced tissue destruction in the observed tissue (30). Notably, β -catenin is considered as a physiological protein guaranteeing integrity of the epithelial tissue formation. However, it is also considered as a tumour-enhancing factor in certain conditions (15). In previous studies, we observed an up-regulation of mural β -catenin concentration through antisense treatment in external auditory canal cholesteatoma *in vitro* (31). We were able to up-regulate β -catenin by incubation with IL-13. Notably, the cytoplasmic concentration of β -catenin did not change in comparison to non-eosinophilic paranasal mucosa samples. As CRS and bronchial asthma have a number of pathological features in common, some authors describe CRS as the 'asthma of the upper airways' (2,5). CD4⁺ Th2 lymphocytes and their cytokine products, especially IL-4, IL-5, IL-9 and IL-13, are essential for generating such asthmatic landmarks as eosinophilic inflammation, hyperplasia and subepithelial fibrosis. Among Th2 cytokines, IL-13 is considered particularly critical. For example some authors report that the local application of IL-13 can induce the asthma phenotype in non-immunized mice (32,33). Notably, glucocorticoids are not able to suppress IL-13-induced airway hyper-responsiveness and goblet cell hyperplasia (32). IL-13 has a variety of proinflammatory effects that are relevant to CRS and bronchial asthma, including the ability to induce IgE production and endothelial cell VCAM-1 expression, whereas vascular cell adhesion molecule (VCAM-1) plays an important role in eosinophil migration in inflamed airways (34). In general, the natural variation in the coding region of IL-13 seems to be an important genetic determinant of susceptibility to allergy (35).

In conclusion, we provide evidence that application of IL-13 increases the β -catenin expression in human eosinophilic paranasal mucosa cell culture. Immunohistochemistry revealed a restriction of β -catenin to the membrane of the cells. In our study, we were able to examine the relations between eosinophilic inflammation and nasal tissue remodelling. Further studies will be necessary to investigate the cellular nature of remodelling inductors and the basic pathways of initial airway inflammation. Thus further molecular links between the cytokines of Th2 lymphocytes and the E-cadherin/ β -catenin complex might be revealed.

Acknowledgements

We would like to thank Ms. P. Prohaska for her excellent technical assistance and Dr C. Weiss, Department of

Biomathematics, University of Heidelberg, for the statistical analysis.

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