Effect of vascular cell adhesion molecule 1 on fibroblasts in human eosinophilic chronic rhinosinusitis

ALEXANDER SAUTER[1], DANIEL BAS-ORTH[1], JENS STERN-STRAETER[1], JAMES BARNES[2], HANNAH SOULSBY[3], KARL HÖRMANN[1] and RAMIN NAIM[4]

[1]Department of Otolaryngology, Head and Neck Surgery, Ruprecht-Karls-University Heidelberg, Faculty of Medicine Mannheim, Mannheim, Germany; [2]Department of General Surgery, University Hospital, Coventry; [3]Warwick Medical School, University of Warwick, Coventry, UK; [4]Department of Otolaryngology, Head and Neck Surgery, University of Homburg/Saar, Homburg/Sarr, Germany

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Abstract. The pathogenesis of eosinophilic chronic rhinosinusitis (ECRS) is still unclear. Paranasal mucosa inflammation is thought to be related to eosinophilic infiltration. This infiltration seems to induce changes in the expression of cell adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1). The E-cadherin-ß-catenin complex maintains the integrity of the epithelium. Down-regulation of ß-catenin and E-cadherin is a pivotal factor for progressive cell growth. This study aimed to assess which cytokines regulate the expression of the adhesion molecule E-cadherin and the multi-functional protein ß-catenin, which plays a key role in cadherin-mediated anchoring in ECRS. Cultured ECRS specimens were incubated with human VCAM-1. After a period of up to 72 h, expression of E-cadherin and ß-catenin was determined using cytokine immunoassay and immunohistochemistry. In ECRS, significant increases in E-cadherin expression were found in fibroblast cell cultures. Stimulation with VCAM-1 did not produce a significant alteration in the expression of the adherens junction protein ß-catenin. In addition, VCAM-1 did not decrease the levels of membrane staining for adherens junction proteins. The selective increase in E-cadherin expression in eosinophilic fibroblast cultures might be explained by a higher concentration of the Th2-type cytokines in these cultures. The tissue remodelling observed during chronic eosinophilic inflammation offers new insight into the pathogenesis of ECRS.

Correspondence to: Dr Alexander Sauter, Universitaets-HNO-Klinik, Theodor-Kutzer-Ufer, D-68135 Mannheim, Germany
E-mail: alexander.sauter@googlemail.com

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Introduction

Chronic rhinosinusitis (CRS) is defined as a disease of the nasal and paranasal sinus mucosa present for more than 3 months with mucosal changes ranging from inflammatory thickening to gross nasal polyps (1,2). CRS remains a significant health problem and is still increasing in prevalence. US data from 1997 indicate a prevalence of approximately 15% in the general population (3). CRS is a heterogeneous group of sinus diseases that may represent different disease entities. As the pathogenesis of chronic rhinosinusitis (CRS) is still unknown, diagnosis of CRS in the USA and Europe is based on symptoms, duration of symptoms, clinical examination, nasal endoscopy and a CT scan. Contributing factors include anatomical variants, atopy, acetylsalicylic acid intolerance and microbial factors (4). Fungal microorganisms have been regularly identified in CRS in varying frequencies and are suspected to initiate and maintain a chronic inflammatory process (5,6). In the response to fungal pathogens, eosinophils and activated mast cells are involved (7,8). The main histological feature of CRS is a persistent underlying eosinophilic inflammation (9). Activated eosinophils contribute to polypoid sinusitis by the release of major basic protein (MBP) in the mucus, epithelial disruption, basement membrane denudation, and through the production of inflammatory cytokines (9-11). During the migration process, eosinophils must pass through the intercellular spaces and tight junctions of epithelial cells (12). Eosinophil infiltration of the epithelium is associated with a decrease in E-cadherin expression (13). Thus, the epithelial cell contact mediated by E-cadherin is loosened due to transepithelial migration of activated eosinophils (14). Cell adhesion plays an important role in tissue morphogenesis and homeostasis and is commonly mediated by cadherins, a family of Ca^{2+}-dependent transmembrane adhesion receptors. The cell-cell adhesion is assumed to be controlled by tyrosine phosphorylation of the adherens junction and desmosome components (15). The level of cadherin expression seems to influence the strength and stability of the adhesion (16). ß-catenin is a 96-kDa cadherin-associated protein that mediates the anchoring of cadherins to actin by binding α-catenin (17).

The endothelial adhesion molecule, vascular cell adhesion molecule 1 (VCAM-1), plays an important role in eosinophil migration into the inflamed airway (18,19). The expression of VCAM-1 is regulated transcriptionally by cytokines and other mediators of inflammation, such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (20). In an animal model, Sephadex beds induced eosinophilia in pulmonary tissue through the contribution of VCAM-1 adhesion pathways (21). Monoclonal antibodies to very late activation antigen-4 (VLA-4) and CD18 caused significant suppression of eosinophilia in the same model (22). Thus the VLA-4/VCAM-1 and CD18/ICAM-1 adhesion pathways may mediate eosinophilia observed in the lungs (23). Another rodent model showed an upregulation of VCAM-1 and ICAM-1 expression on endothelial cells in the initial stages of eosinophil migration into lung tissues (24). Dexamethasone abolished this upregulation of VCAM-1 and ICAM-1 expression in the lung (24).

In accordance with similarities observed in the pathogenesis of chronic airway inflammation and rhinosinusitis, we hypothesized that incubation of ECRS tissue with VCAM-1 might alter the expression of ß-catenin and E-cadherin in paranasal fibroblast cultures.

Materials and methods

Tissue collection and culture of human paranasal mucosa. 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 use of the tissue samples of the resected paranasal mucosa and turbinates, and the study was approved by the Ethics Committee of the Faculty of Medicine, Mannheim, University of Heidelberg, Germany. Surgery was followed by pathological examination of the samples. The samples were diagnosed as eosinophilic and non-eosinophilic CRS (NECRS) depending on the amount of eosinophilic granulocytes in the samples. We set up a fibroblast culture for each sample of mucosa from either paranasal sinus or 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 primary culture of fibroblast cells, the suspension was added onto mitomycin-treated (23.9 μM) 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% CO_2 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 cells were further cultured in fibroblast growth medium (Fisher Scientific Co., Pittsburgh, PA, USA). Cells were passaged by trypsinisation (0.1% trypsin and 0.02% EDTA dissolved in PBS, 5 min, 37˚C).

Cytokine immunoassay. Cell culture supernatants were collected in sterile test tubes and stored at -20˚C until further use. The concentrations of the two examined adherens junction proteins were determined by an ELISA technique (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against recombinant cytokines. According to the manufacturer's directions, each ELISA assay measured 100 μl of supernatant. The cells were grown in 96-well plates (Part 890218) with 12 strips of 8 walls coated with mouse antibody against either ß-catenin or E-cadherin. After 0, 24, 48 and 72 h of incubation with 300 ng/ml of human recombinant VCAM-1 (catalog no. 809-VR, R&D Systems, Minneapolis, MN, USA), the expression of the ß-catenin and E-cadherin protein in the supernatants of the VCAM-1 and untreated culture cell lines was analysed.

Immunohistochemistry. Immunohistochemical analysis was performed using a monoclonal mouse anti-human antibody directed against ß-catenin (C19220, Transduction Laboratories, Lexington, KY, USA) and E-cadherin (M3612, Dako, Hamburg, Germany). Immunostaining was performed using the alkaline-phosphatase-anti-alkaline phosphatase method (APAAP). The specimens were transferred onto glass slides and air-dried overnight at 37˚C. Sections received a microwave pre-treatment, which required boiling for 15 min at 600 W using citrate buffer for ß-catenin or E-cadherin. The following steps were performed by an automated staining system, Dako TechMate 500. Sections were incubated with the primary antibody solution for 25 min at room temperature, using a working dilution 1:300 for the antibody and the adherens junction protein respectively. Slides were rinsed once in buffer (Buffer Kit, Dako). Immunoreaction was demonstrated with the Dako ChemMate Detection Kit (APAAP, Mouse, code no. K 5000), according to the specifications of the manufacturer. Sections were incubated with the chromogen alkaline-phosphatase-substrate (Neufuchsin, Dako) for 20 min at room temperature. Finally, sections were counterstained using Mayer's hematoxylin for 3 min, dehydrated in graded ethanol, and coverslipped. Negative controls used all reagents except the primary antibody.

Analysis of E-cadherin/ß-catenin immunostaining. The rates of expression were analysed semiquantitatively. The number of positively marked epithelial cells was graded as ranging from 0 (no positive cells), 1 (<20% positive cells), 2 (20-50% positive cells) to 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, 1/II, 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 ß-catenin and E-cadherin. To ensure the observer reliability of the assessment, the specimens were blindly assessed by two independent reviewers unaware of all clinical data. Differences between the two investigators were resolved by consensus.

Statistical analysis. The statistical analysis was performed using the program SAS (SAS/STAT; version 8, SAS Institute Inc., Cary, NC, USA). The significance of normally distributed samples was analysed by using the Student's t-test in order to evaluate the effect of VCAM-1. A probability of $p<0.05$ was

Table I. Incubation of paranasal ECRS fibroblast culture with 300 ng/ml VCAM-1.

Incubation time (h)	E-cadherin (ng/ml)		ß-catenin (ng/ml)	
	Verum[a]	Control[a]	Verum[a]	Control[a]
8	0.0770/0.0331	0.1690/0.0263	15.5655/2.7509	13.1075/1.4217
24	0.1860/0.0220	0.1843/0.0226	16.2835/3.1673	15.7930/4.1708
48	0.2828/0.0701	0.2188/0.0568	19.2293/3.4534	15.6880/3.2180
72	0.3240/0.1438	0.2163/0.0674	21.6153/3.9516	16.0755/3.9297
	Adhesion molecule - percentage of total protein (%)			
8	0.00052	0.00053	0.0051	0.0042
24	0.00037	0.00055	0.0033	0.0047
48	0.00056	0.00092	0.0038	0.0075
72	0.00079	0.00041	0.0050	0.0030

[a]Median/standard deviation (ng/ml). ECRS, eosinophilic chronic rhinosinusitis; VCAM-1, vascular cell adhesion molecule-1. Fibroblast culture with (Verum) and without (Control) VCAM-1 incubation.

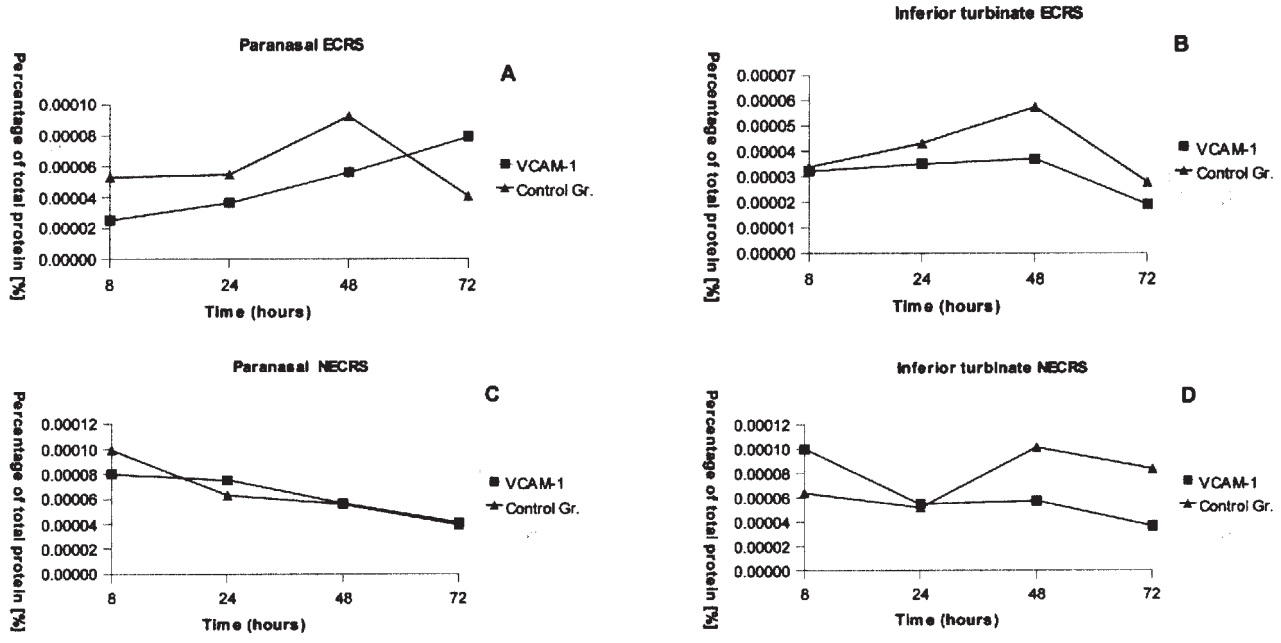

Figure 1. Effect of VCAM-1 on E-cadherin expression. Paranasal (A) and inferior turbinate (B) ECRS and NECRS (C and D) fibroblasts were cultured with 300 ng/ml VCAM-1. Non-stimulated cultures were also studied (control). After 8, 24, 48 and 72 h of culture, supernatants were collected, and E-cadherin expression was determined by the use of enzyme-linked immunosorbent assay (ELISA).

considered statistically significant. The influence of VCAM-1, incubation time, tissue origin and comparison of the various groups were analyzed using the Wilcoxon signed rank test for paired observations (SAS/STAT).

Results

E-cadherin: Incubation to a maximum of 72 h with 300 ng/ml VCAM-1. After 8 h of incubation, 0.077 ng/ml E-cadherin was detected in the supernatants of the treated fibroblasts, whereas in the controls 0.169 ng/ml was found. After 24 and 48 h, 0.186 and 0.2828 ng/ml E-cadherin respectively were measured in the treated cell cultures (Table I). The percentage of E-cadherin of the total protein concentration increased slightly from 0.0000527% after 8 h to 0.00006% (EP 0.00032%) after 72 h in the treated fibroblast cultures. On the other hand, the untreated cultures showed a decrease from 0.000076 to 0.00004% after incubation for 72 h (Fig. 1A-D). The Wilcoxon signed rank test revealed a significant difference in the E-cadherin expression in the treated fibroblast cultures compared to the controls without incubation with VCAM-1 (p=0.0391). Further statistical analysis was performed in order

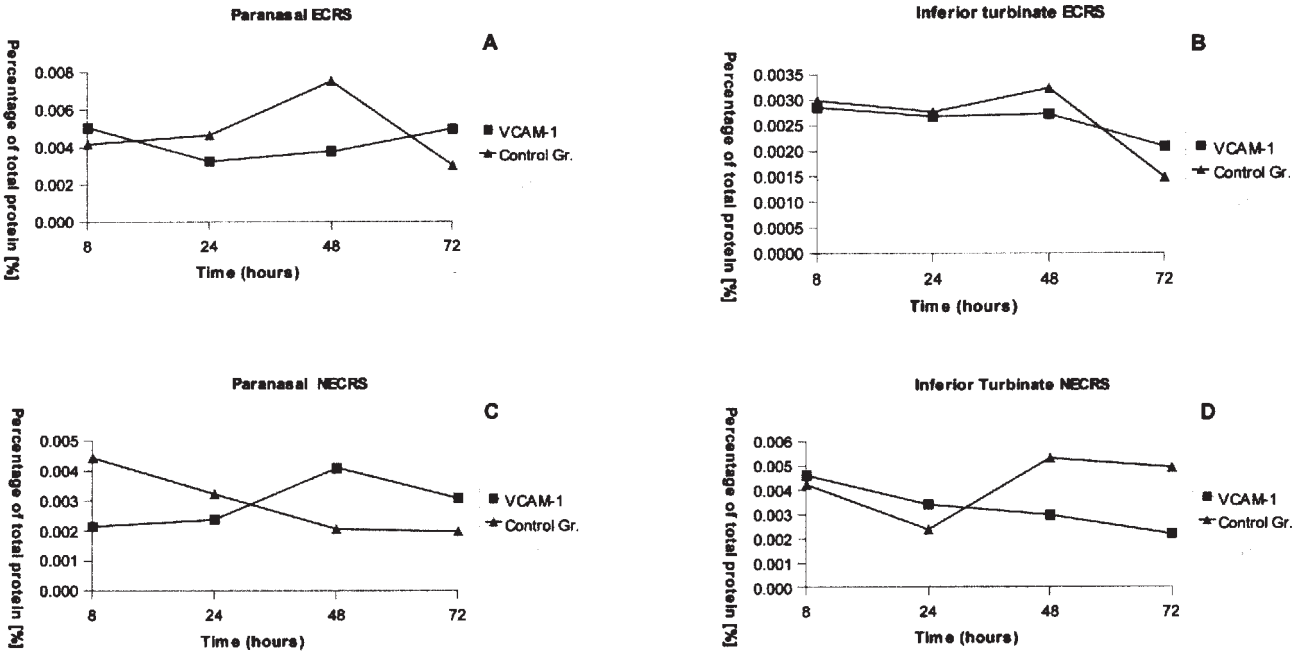

Figure 2. ß-catenin expression in paranasal (A) and inferior turbinate (B) ECRS and NECRS (C and D) fibroblasts stimulated by 300 ng/ml VCAM-1. After 8, 24, 48 and 72 h, ß-catenin expression was analysed by the use of ELISA. Non-stimulated cultures were also studied (control).

to evaluate the influence of tissue origin (mucosa of sinus or inferior turbinate), eosinophils and hours of incubation. The analysis revealed no significant influence concerning the E-cadherin expression in non-eosinophilic tissue after VCAM incubation (p=0.5095). Nevertheless, eosinophils did have a significant influence on the level of E-cadherin expression in the treated fibroblast culture (p=0.0087).

ß-catenin: Incubation to a maximum of 72 h with 300 ng/ml VCAM-1. After an 8-h incubation with VCAM-1, ß-catenin was measured at 15.5655 ng/ml (control 13.1075 ng/ml) in fibroblast cultures of eosinophilic paranasal mucosa. After 72 h, treated fibroblast eosinophilic paranasal cultures showed 21.6153 ng/ml (control 16.0755 ng/ml) of ß-catenin (Table I). The percentage of ß-catenin of the total protein concentration dropped from 0.0051% after 8 h to 0.0050% after 72 h in the treated fibroblast cultures (Fig. 2A-D). The Student's t-test failed to show significant differences in ß-catenin expression in the treated eosinophilic cultures compared to the untreated control cultures (p=0.3391). The Wilcoxon signed rank test showed no significant influence of VCAM-1 on the ß-catenin concentration in all the treated cultures compared with their controls (p=0.6769).

Immunohistochemistry. After 48 h of incubation with VCAM-1, reactivity to ß-catenin was observed at the membranes of ECRS cells, whereas the untreated ECRS cells (control) showed a reduced reactivity to ß-catenin at the membranous borders. The staining of E-cadherin was reduced compared with the immunostaining of ß-catenin. In addition, the immunostaining was not absolutely restricted to the membrane of the cells. Comparing the immunoreactivity of the basal layer of non-eosinophilic paranasal mucosa and eosinophilic paranasal mucosa in CRS, there was neither a difference in E-cadherin nor a difference in ß-catenin expression. However, the eosinophilic paranasal mucosa samples did show a high mural expression of ß-catenin. The results are presented in Fig. 3A and B and Table II.

Discussion

ß-catenin affects cell adhesion in epithelial tissue by its transduction via the intracellular component α-catenin and by arranging the binding of cytosolic filaments and the extracellular component E-cadherin (14). Cell adhesion plays an important role in tissue morphogenesis. The level of cadherin expression seems to influence the strength and stability of the adhesion (15). E-cadherin and ß-catenin play an important role in the maintenance of cell integrity and thus represent two major components of the extracellular matrix (ECM). ECM components, for example fibroblasts, can produce eotaxin through a synergistic action of interleukins and tumour necrosis factor α (22). The increased expression of these pro-inflammatory cytokines are known to be associated with increased permeability of the epithelium, which implies that alteration of cell-cell contacts may have immunomodulatory consequences. The importance of ECM remodelling has already been investigated in lower airway disease. In chronic sinus disease, similar types of fibrosis can be found in the submucosa. The amount of subepithelial collagen deposition has been found to be greater in patients suffering from ECRS compared with normal tissue (23). In ECRS with polyp formation, frequent epithelial damage and a thickened basement membrane have also been observed (24). This is demonstrated in our study by the high mural expression of ß-catenin in eosinophilic paranasal cultures. Epithelial thickening and eosinophilia have been found even in inferior turbinates located at a distance from the polyps (25). Eosino-

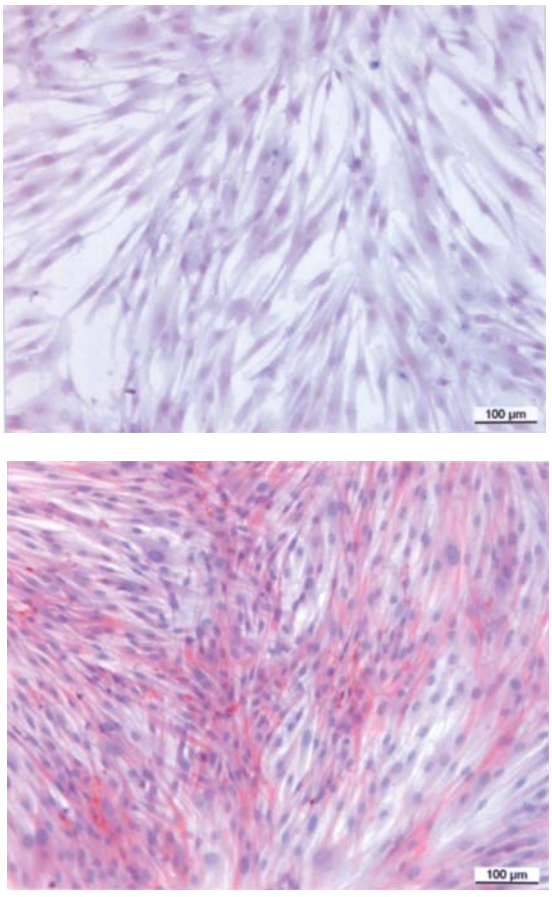

Figure 3. Immunohistochemical staining and phase contrast light microscopy of human fibroblasts in eosinophilic chronic rhinosinusitis (ECRS) cell culture. (A) Anti-ß-catenin immunohistochemical staining of paranasal fibroblasts after 8 h (x100) and (B) 72 h (x200) of incubation with 300 ng/ml VCAM-1.

Table II. Immunoreactivity score: Grading of ECRS and NECRS after detection of E-cadherin and ß-catenin in primary (0 h) and cultured (24 and 72 h) fibroblasts.

		Immunoreactivity (n=4/4)	
Tissue	Score[a]	ECRS/Control	NECRS/Control
Paranasal tissue			
0 h/ß-catenin	0-1/II	x[b]/4	x[b]/4
	2/I-3/II	x[b]/0	x[b]/0
0 h/E-cadherin	0-1/II	x[b]/4	x[b]/4
	2/I-3/II	x[b]/0	x[b]/0
24 h/ß-catenin	0-1/II	0/4	1/4
	2/I-3/II	4/0	3/0
24 h/E-cadherin	0-1/II	3/4	3/4
	2/I-3/II	1/0	1/0
72 h/ß-catenin	0-1/II	1/3	3/4
	2/I-3/II	3/1	1/0
72 h/E-cadherin	0-1/II	2/3	0/4
	2/I-3/II	2/1	4/0
Inferior turbinate			
0 h/ß-catenin	0-1/II	x[b]/4	x[b]/4
	2/I-3/II	x[b]/0	x[b]/0
0 h/E-cadherin	0-1/II	x[b]/4	x[b]/4
	2/I-3/II	x[b]/0	x[b]/0
24 h/ß-catenin	0-1/II	4/4	4/4
	2/I-3/II	0/0	0/0
24 h/E-cadherin	0-1/II	4/4	4/4
	2/I-3/II	0/0	0/0
72 h/ß-catenin	0-1/II	1/1	3/4
	2/I-3/II	3/3	1/0
72 h/E-cadherin	0-1/II	3/4	4/4
	2/I-3/II	1/0	0/0

[a]Immunohistochemical score: 0-1/II, negative to low expression of E-cadherin/ß-catenin; 2/I-3/II, strong expression of E-cadherin/ß-catenin. ECRS, eosinophilic chronic rhinosinusitis; NECRS, non-eosinophilic chronic rhinosinusitis. Incubation, 300 ng/ml VCAM-1. [b]Primary cell culture without VCAM-1 incubation.

philic inflammation seems to play a central role in ECRS. VCAM-1 and certain interleukins have been reported to be the most relevant pathogenic proteins resulting in ECRS. The cytokine-dependent induction of VCAM-1 is regulated at the gene level by the activity of transcription factors such as NF-κB, AP-1, specificity protein-1 (SP-1), IFN regulatory factor-1 (IRF-1), and GATA (26). It has been described that Th2 cells *in vitro* are capable of sustaining high-affinity adhesive interaction with VCAM-1 (27). This supports the hypothesis that VCAM-1 might be involved in Th2-type cell migration. In addition, VCAM-1 seems to play a central role in the selective migration of eosinophils (28). Del Maschio *et al* reported that the engagement of adhesion molecules may be crucial in triggering intracellular signals in endothelial cells and thus induce an upregulation of the cadherin-complex disorganisation (29). We did not observe an increasing disorganisation by incubation with 300 ng/nl VCAM-1, but an increased expression of E-cadherin by the fibroblast cultures was observed in the immunoassay. Furthermore, we showed with immunostaining that E-cadherin and ß-catenin are up-regulated on ECRS cells, which may cause the development of polypoid structures. The upregulation of E-cadherin in ECRS cultures after incubation with VCAM-1 might be associated with dedifferentiation and building of polypoid sinus mucosa structures. Furthermore, changes in the distribution of cell adhesion molecules are most likely responsible for the damage to the epithelial barrier and thus contribute to the increase in mucosal permeability (30). The blockage of E-cadherin and ß-catenin by antibodies has been found to induce tissue destruction and dissociation of cultured tissue (31). Kobayashi *et al* reported that the immuno-reactivity of cell adhesion molecules was lost specifically at regions of eosinophil adhesion (13). This is in accordance with our observations that immunostaining of E-cadherin was reduced compared to staining of ß-catenin in histochemistry. This might also be explained by an increasingly enhanced detachment of the cell layers with an extended culture course. Furthermore, we observed an enhanced distribution

of E-cadherin at the sites where the cells attached to the culture but not at the intercellular junctions between adjoining cells. Thus, the upregulation of E-cadherin observed in the immunoassays, might be explained by alterations of the fibroblast morphology and function of the cell barrier. ß-catenin is considered to be a physiological protein, but in certain conditions ß-catenin does act as a tumour-enhancing factor (14). We found that the cytoplasmatic concentration of ß-catenin did not change in comparison to untreated cultures. Several interleukins are capable of mediating and inducing ECM remodelling in murine models of allergic asthma (32). Kobayashi *et al* reported a decrease in E-cadherin expression after incubation of nasal epithelial cells with IL-5 (13). In pulmonary tissue, VCAM-1 can even mediate infiltration with eosinophils (20). Although the trafficking of eosinophils, their transmigration through vascular walls and the release of cytokines is facilitated by adhesion molecules, we observed only an increase in E-cadherin expression by sole incubation with VCAM-1. Considering the above, we believe that the effect of the eosinophils on the expression of E-cadherin and ß-catenin is likely not only to be mediated by cytokines and adhesion molecules, but also by the cell number and level of eosinophil activation.

In conclusion, the results of this study demonstrate that the remodelling of the ECM in ECRS seems to be based on modifications in cell adhesion molecules such as ß-catenin, E-cadherin and VCAM-1. Such changes in the distribution of cell adhesion molecules in the sinus mucosa could trigger the damage and dissociation of the epithelial barrier, thus contributing to alterations in mucosal permeability and pseudocyst formations. Targeting of such alterations in adhesion molecules seems to present possible therapeutic strategies for the future, but further studies are necessary.

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