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Regulation of Matrix Metalloproteinases (MMP)-2/-9 Expression in Eosinophilic Chronic Rhinosinusitis – Cell Culture by Interleukin-5 and -13 ?

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Abstract. *Background: Eosinophils are a prominent immunological feature of chronic rhinosinusitis (CRS). Cytokines in the respiratory mucosa may be the key to upper airway pathophysiology. Matrix metalloproteinases (MMP) represent an entire group of Zn^{2+} dependent endopeptidases with the potential to alter the extracellular matrix (ECM). In this study epithelial cultures of CRS were treated with interleukin (IL)-5 or IL-13 and subsequent levels of metalloproteinases were determined. Materials and Methods: The cells for CRS culture were obtained from patients undergoing functional endoscopic sinus surgery. After 8-72 hours incubation with 0.2-0.4 ng/ml IL-5 or 3-6 ng/ml IL-13, the expression of the MMP-2 and -9 in the CRS cultures was analysed. Results: After 72 hours incubation with IL-5, the relative levels of MMP-2 showed no significant alteration in protein expression in comparison with the control groups. Incubation with IL-13 revealed a statistically insignificant decrease of the relative MMP-9 expression in ECRS compared to the control group ($p>0.1$). Conclusion: Alterations of MMP-2 and -9 expression may play a role in ECRS, but the association with IL-5 and IL-13 remains unclear.*

Chronic rhinosinusitis (CRS) is defined as a disease of the nasal and paranasal sinus mucosa present for longer than 3 months duration and associated with mucosal changes ranging from inflammatory thickening to nasal polyps (1). Patients with CRS have long-term nasal congestion, thick mucous production, anosmia and acute intermittent exacerbations secondary to bacterial infection. In addition, their quality-of-life is more severely impaired than patients with congestive heart failure (2). The diagnosis is based upon the presence of typical symptoms together with clinical manifestations, but the pathogenesis remains unclear.

CRS reveals frequent epithelial damage, a thickened basement membrane and oedematous to fibrotic tissue. Infiltration of eosinophils is a characteristic feature of CRS, and the formation of pseudocysts has been frequently reported. Consistent with this pathological fact is the reporting of increased aquaporin-1 water channel expression in CRS tissue (3). Matrix metalloproteinases (MMP) are responsible for extracellular matrix (ECM) degradation in embryonic development and tissue morphogenesis (4). They are also involved in various pathological processes such as inflammation or tumour invasion (4, 5). Recently, an imbalance between MMPs and their natural inhibitor, tissue inhibitor metalloproteinase (TIMP)-1 has been reported in CRS. This imbalance could induce a local increase of ECM degradation and the formation of pseudocysts (5). Like neutrophils, mast cells can express MMPs and interact with the ECM (6). This is supported by the fact that MMP-2 is part of the pathogenesis of CRS (7, 8).

Recent studies have reported that MMP deficiency, especially low levels of MMP-8 and MMP-9, can promote airway inflammation (9, 10). It has also been reported that levels of gelatinase B (MMP-9) and TIMP-1, are elevated in CRS (5). In chronic asthma, eosinophils are the source of MMP-9 (11). Not only have MMP-2 and MMP-9 been found in CRS with polyps, but the extent of MMP-9 expression could predict healing after sinus surgery (5, 8). Several cytokines are known to affect the production of MMP-2, MMP-9 and TIMP-1. In airway epithelium, IL-8 seems to potentially act as an initial activator of MMP-9 during airway epithelial regeneration (12).

In a study of the relationship between MMP-1 and TIMP-1 enzymes and the helper T cell (TH) 1 and TH 2 cytokine

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system, a correlation between the serum levels of interleukin (IL)-5 and IL-8 and the amount of TIMP-1 was found (13). Futhermore *in vitro* studies in Ewing's sarcoma have shown that enhanced MMP-9 expression resulted in decreased levels of E-cadherin (14). It has been reported that the level of cadherin expression influences the strength of adhesion (15). E-cadherin is a cell surface protein responsible for the cell integrity and morphogenesis of epithelial tissues. The maintenance of organised tissue is established by cell-to-cell mediated adhesion. Thus contradicting reports of increased MMP/TIMP-1 levels in association with better clinical outcomes after functional endoscopic sinus surgery might reflect disturbances in tissue repair mechanisms (16).

Damage to the respiratory epithelium, which impedes the barrier function of mucosa and affects its permeability, is known to contribute to the pathogenesis of airway diseases (17). The damage to the epithelium closely correlates with the degree of eosinophil infiltration. Eosinophil products are known to cause epithelial damage and submucosal oedema (18). The key role of nasal polyposis seems to be the recruitment, activation and survival of eosinophils (19). Mediators such as IL-5 and eotaxin are considered to be key factors for eosinophilic accumulation and activation in nasal polyps (20). Eosinophil migration is dependent on the expression of cytokines, chemokines, and adhesion molecules (21). Resident cells, for example, T lymphocytes, release IL-1β, IL-4 and TNF-α and thus enhance the endothelial adhesion molecules (22-24), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin. This enhancement results in a stronger leukocyte-endothelial adherence and transendothelial migration of leukocytes along a chemotactic gradient. Increased local expression of ICAM-1, VCAM-1 and E-selectin has been demonstrated after experimental nasal and bronchial allergen challenge (25, 26). The cytokine pattern in chronic polypoid sinusitis often shows neither a T helper 1 (TH-1) nor a TH-2 type predominance, because IL-4, IL-5, IL-12 and IFN-γ have all been shown to be elevated in nasal polypoid tissue without influencing the atopic status of an individual (27). In paranasal polyposis, IL-5 expression has been observed in polyps and inferior turbinates, whereas no detectable values of IL-5 were found in the serum of the same patients (28).

IL-5 and IL-13 play a central role in CRS and are known to have eosinophil-selective chemoactivities. An *in vitro* study was therefore designed to determine the influence of these cytokines on MMPs in CRS and acute rhinosinusitis.

Materials and Methods

Tissue collection and culture of human chronic rhinosinusitis epithelial cells. All the CRS cells were obtained from 4 patients suffering from CRS and who underwent 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 the patients to take tissue samples of the resected paranasal mucosa and turbinates. This study was approved by the Ethics committee of the Faculty of Medicine, Mannheim, University of Heidelberg, Germany. After surgery, the tissue samples were examined by a pathologist and diagnosed as eosinophilic and non-eosinophilic CRS according to the levels of eosinophilic granulocytes within the samples. An epithelial culture was set up for each sample of paranasal sinus mucosa and for each inferior nasal turbinate. After the 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. To create a primary culture of epithelial cells, each suspension was added onto a mitomycin-treated (23.9 μM) human fibroblast monolayer, 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, insulin, triiodothyronine hydrocortisone, epidermal growth factor, cholera toxin and penicillin or 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 sinus epithelial cells were further cultured in keratinocyte growth medium (KGM, Clonetics, San Diego, CA, USA) without serum. The cells were passsaged by trypsinisation (0.1% trypsin and 0.02% EDTA dissolved in PBS, 5 min, 37˚C).

ELISA principle. The Quantikine MMP-2 (catalogue number DMP 200) and MMP-9 (catalogue number DMP 900) immunoassays from R&D Systems (Minneapolis, MN, USA) were used to determine the exact concentration of MMP-2 and -9 in the cell culture supernatants after homogenization. The assays employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific to either MMP-2 or MMP-9 was pre-coated onto a microplate. The standards and samples were pipetted into the wells. The MMPs present were bound by the immobilised antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific to either MMP-2 or MMP-9 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour was allowed to develop in proportion to the amount of peptide bound in the initial step. The colour development process was halted and the intensity of the colour was measured.

The cells were grown in 96-well plates (Part 890218) with 12 strips of 8 walls coated with mouse antibody against either MMP-2 or -9. After 8, 24, 48 or 72 hours of incubation with either 0.2 - 0.4 ng/ml human recombinant IL-5 (catalogue number 205-IL) or 3 or 6 ng/ml IL-13 (catalogue number 213-IL) from R&D Systems, the expression of the MMPs in the supernants of the treated and untreated culture cell lines was analysed.

Immunohistochemical analysis. Immunohistochemistry (IHC) was performed using monoclonal antibodies against MMP-2 (clone 42-5D11) and MMP-9 (clone 56-2A4) (Calbiochem La Jolla, CA, USA) after 24 h and 72 h of incubation with IL-5 or IL-13. The staining was evaluated as previously described (29). The results of the immunohistochemically obtained rates of expression were analysed semiquantitatively. The number of positively marked epithelial cells was graded as ranking from 0 (no positive cells), 1 (<10% positive cells), 2 (10-50% positive cells) to 3 (>50% positive cells). The intensity was noted as I (faint) or II (strong).

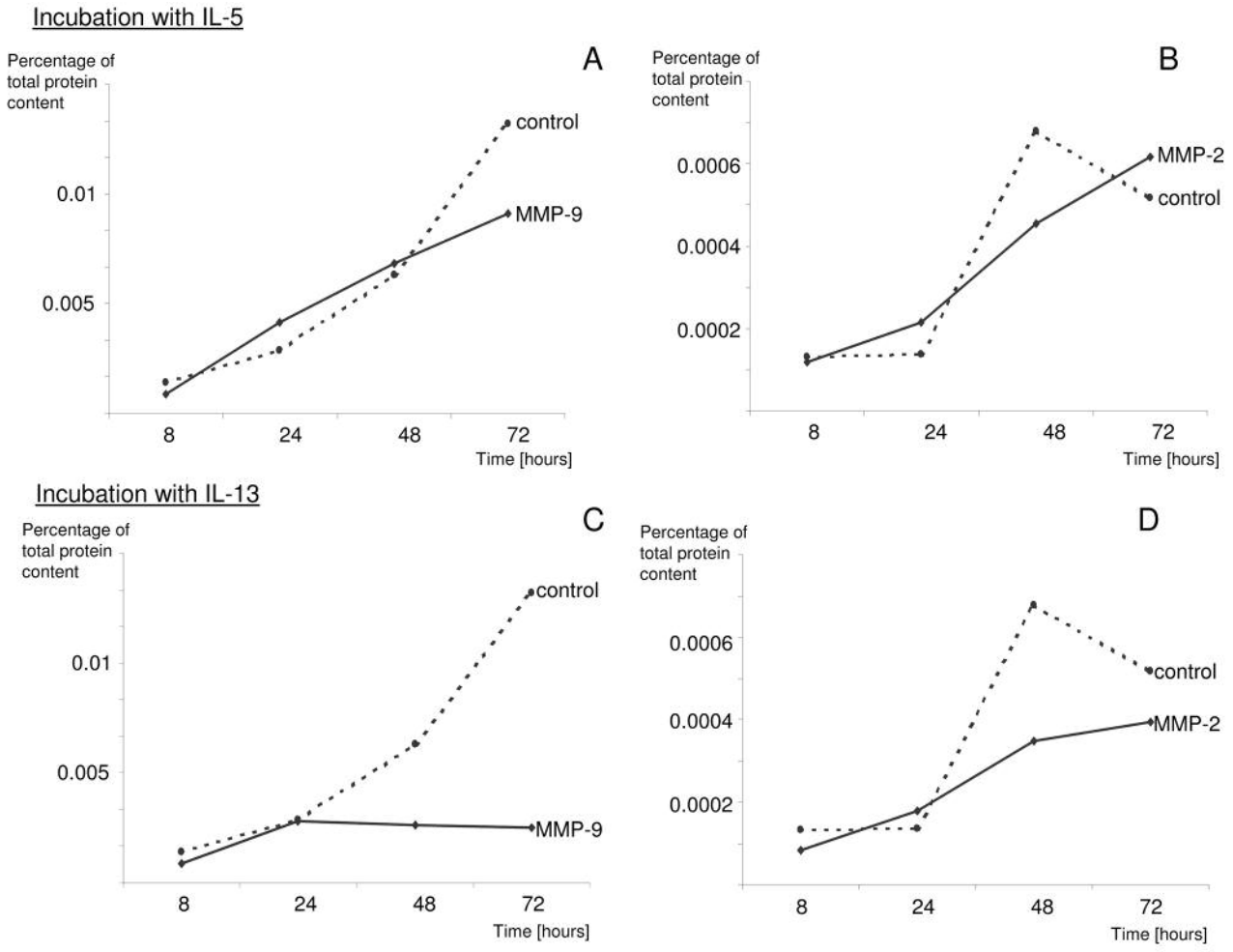

Figure 1. *Enzyme-linked immunosorbent assay (ELISA)-measurements: comparison of relative expresssion of matrix metalloproteinase (MMP)-9 (A) and -2 (B) after IL-5 (0.4 ng/mL) incubation, expression of MMP-9 after incubation with IL-13 (6 ng/mL) (C), and MMP-2 after incubation with IL-13 (6 ng/mL) (D). The control group was not incubated with IL-5 or IL-13.*

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 1/II were classified as negative or low expression and 2/I to 3/II as high expression of the MMP-9 and -2. 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 SAS programme (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 cytokine effect on metalloproteinase release. A probability of $p<0.001$ was considered statistically significant. The influence of incubation time, rising concentrations of IL-13 and -5 and comparison of the various groups were analyzed using the general linear model (GLM) procedure (SAS/STAT).

Results

Immunoassay (ELISA). After incubation with 0.2 ng/mL IL-5, the majority of the eosinophilic CRS cultures had lower expressions of MMP-2 (mean: 0.853 ng/mL, standard deviation 0.991 ng/mL) compared to the cultures of non-eosinophilic CRS (MMP-2/IL-5: mean 1.471 ng/mL, standard deviation 1.778 ng/mL). The eosinophilic cultures had a higher expression of MMP-9 (mean 12.928 ng/mL, standard deviation 10.915 ng/mL) than the non-eosinophilic cultures (MMP-9/IL-5: mean 4.206 ng/mL, standard deviation 5.742 ng/mL) after incubation with rising concentrations (0.2 ng to 0.4 ng/mL) IL-5. Incubation with 6 ng/mL IL-13 decreased the MMP-9 concentrations in the eosinophilic CRS tissue (MMP-9: mean 8.456 ng/mL, standard deviation 5.113 ng/mL) compared to the non-

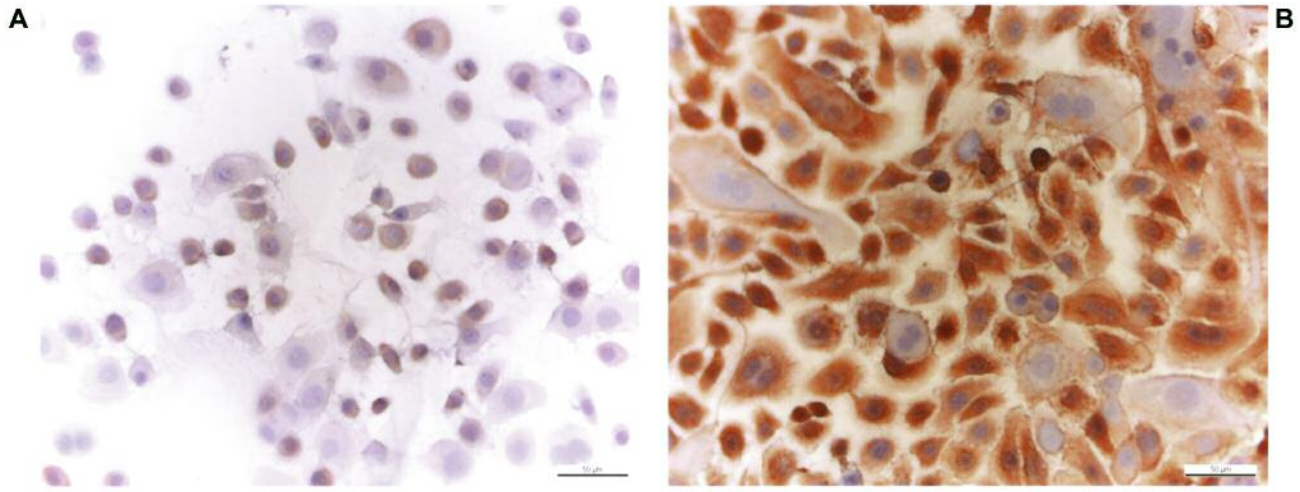

Figure 2. *Immunhistochemistry. Representative matrix metalloproteinase (MMP)-2 staining of suprabasal layers, but also connective tissue (A). MMP-9 staining of surface epithelium, especially in suprabasal layers (B) (magnification: 200-fold).*

eosinophilic tissue (MMP-9/IL-13: mean 2.661 ng/mL, standard deviation 2.703 ng/mL). In contrast, incubation with IL-13 did not induce a higher MMP-2 expression in the eosinophilic tissue (MMP-2: mean 1.001 ng/mL, standard deviation 1.223 ng/mL) compared to the non-eosinophilic tissue (MMP-2/IL-13: mean 2.623, standard deviation 2.469 ng/mL). Comparing the treated samples with the control samples after incubation with 0.4 ng/mL IL-5 showed that the MMP-9 protein concentration was not significantly different in eosinophilic CRS (p=0.1018) and in non-eosinophilic CRS (p=0.5751). Variance analysis revealed that in eosinophilic CRS, MMP-9 expression was significantly determined by the length of incubation with IL-5 (p<0.0001) compared to the control (p=0.0042). In terms of MMP-9 expression, the incubation had a significant influence on both the eosinophilic CRS and the control (p=0.0002). The statistical GLM procedure showed that MMP-9 expressions were significantly increased in eosinophilic CRS compared with the controls (p=0.0021). MMP-9 concentrations in eosinophilic paranasal tissue (p=0.0003) and eosinophilic inferior turbinbate (p<0.0001) were significantly elevated compared to the non-eosinophilic cultures after incubation with IL-5.

In contrast, the comparison of the relative amounts of MMPs in eosinophilic paranasal tissue were not significantly different than in the various controls. Incubation of eosinophilic paranasal tissue with IL-13 could neither achieve a significant difference in MMP-9 expression (p=0.0305) nor in MMP-2 expression (p=0.0948). Finally, with respect to the relative percentage of total protein expression, IL-5 incubation did not result in significant differences in MMP-2 (p=0.4340) and MMP-9 (p=0.6912) in eosinophilic paranasal tissue (Figures 1 A-D).

Immunohistochemistry of MMP-2 and MMP-9. Cytoplasmic staining of MMP-2 was observed in the suprabasal layers, but also in the connective tissue. Submucosal glands and vascular structures were marked MMP-2-positive as well (Figure 2A).

The surface epithelium, especially the suprabasal layers, stained MMP-9-positive (Figure 2B). The staining of inflammatory cells was elevated in the paranasal tissue compared with staining of cultures of the inferior turbiate. The staining showed a maximum around blood vessels. In the eosinophilic paranasal tissue, MMP-9 staining was increased within the epithelium, this increase was not statistically significant compared to the controls. The differences between eosinophilic and non-eosinophilic culture were also not statistically significant for MMP-2 after incubation with IL-5 (Table I) or for MMP-9 after incubation with IL-13 for 72 hours (Table II). The staining of the MMPs showed a strong relation to the length of incubation.

Discussion

The etiology of ECRS is still unknown. The development of CRS seems to be a complex multifactorial process. Mononuclear cells, consisting of T and B lymphocytes and activated eosinophils, are prominent in the sinus mucosa and mucus of patients suffering from CRS. Th2 cytokines, for example IL-4, IL-5, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF), seem to play a pivotal role in the cellular recruitment of inflammatory infiltrates such as eosinophils (30). In the last ten years several studies have described local production of inflammatory mediators in patients with

Table I. *Immunoreactivity score (IRS): grading of eosinophilic and non-eosinophilic CRS/MMP-2.*

Tissue	Immunohistochemical Score *	Immunoreactivity n=4	
		ECRS	NECRS
Paranasal tissue			
0 h	0-1/II	2	4
	2/I-3/II	2	0
24 h	0-1/II	2	2
	2/I-3/II	2	2
24 h/ IL-5	0-1/II	4	2
	2/I-3/II	0	2
72 h/ IL-5	0 - 1/II	0	0
	2/I-3/II	4	4
24 h/ IL-13	0-1/II	2	0
	2/I-3/II	2	4
72 h/IL-13	0-1/II	0	0
	2/I-3/II	4	4
Inferior turbinate			
0 h	0-1/II	4	2
	2/I-3/II	0	2
24 h	0-1/II	2	0
	2I-3/II	2	4
24 h /IL-5	0-1/II	4	2
	2/I-3/II	0	2
72 h /IL-5	0-1/II	0	0
	2/I-3/II	4	4
24 h /IL-13	0– 1/II	4	0
	2/I-3/II	0	4
72 h /IL-13	0– 1/II	0	0
	2/I-3/II	4	4

*0-1/II: Negative to low expression of MMP-2; 2/I-3/II: strong expression of MMP-2; ECRS: eosinophilic chronic rhinosinusitis, NCRS: non-eosinophilic chronic rhinosinusitis; Incubation: 0.4 ng/mL IL-5, 6 ng/mL IL-13.

Table II. *Immunoreactivity score (IRS): grading of eosinophilic and non-eosinophilic CRS/MMP-9.*

Tissue	Immunohistochemical Score *	Immunoreactivity n=4	
		ECRS	NECRS
Paranasal tissue			
0 h	0-1/II	2	2
	2/I-3/II	2	2
24 h	0-1/II	2	2
2/I-3/II	2	2	
24 h/ IL-5	0-1/II	2	2
	2/I-3/II	2	2
72 h/ IL-5	0-1/II	0	0
	2/I-3/II	4	4
24 h/ IL-13	0-1/II	2	2
2/I-3/II	2	2	
72 h/IL-13	0-1/II	2	0
	2/I-3/II	2	4
Inferior turbinate			
0 h	0-1/II	2	0
2/I-3/II	2	4	
24 h	0-1/II	4	0
2/I-3/II	0	4	
24 h /IL-5	0-1/II	2	2
	2/I-3/II	2	2
72 h /IL-5	0-1/II	0	0
	2/I-3/II	4	4
24 h /IL-13	0-1/II	4	0
	2/I-3/II	0	4
72 h /IL-13	0-1/II	0	0
	2/I-3/II	4	4

*0-1/II: Negative to low expression of MMP-9; 2/I-3/II: strong expression of MMP-9; ECRS: eosinophilic chronic rhinosinusitis, NCRS: non-eosinophilic chronic rhinosinusitis; Incubation: 0.4 ng/mL IL-5, 6 ng/mL IL-13.

CRS. Eosinophil inflammation seems to play a central role in CRS, especially in ECRS. IL-13 and IL-4 are Th2-type cytokines and are expressed in eosinophilic inflammations (31). IL-13 itself, even in the absence of IgE and eosinophila, is able to mediate and introduce ECM remodeling in murine models of allergic asthma (32). Former studies have reported an enhancement of IL-5 in patients suffering from nasal polyposis (1). IL-5 belongs to the chemokines of the β-chemokine family (CC), which are generally chemoattractant for T lymphocytes, monocytes and natural killer (NK) cells. The group of CC chemokines activates and attracts eosinophils. In nasal polyposis IL-5 was found to be one of the key factors for eosinophilic accumulation and activation (20). Emphasizing the importance of IL-5 in the eosinophilic inflammation type of CRS. While immunohistochemical analysis revealed IL-5 positive eosinophils in nasal polyposis, it did not detect IL-5 positive cells.

However, in the present study, the relative MMP-2 and MMP-9 concentrations after incubation with IL-5 in ECRS culture were at the same level as in the controls. This observation was supported by earlier reports. Chen *et al*. observed a higher expression of IL-5 and MMP-9 in nasal polyps, but they failed to detect a correlation between expression of IL-5 and MMP-9 (33). MMP-9 gene deletion has been reported to be associated with decreased peribronchial eosinophilic inflammation (34). MMP-2 and MMP-9 regulate the formation of transepithelial C-C chemokine gradients, which conduct the extravasated inflammatory cells to the airway lumen where they are cleared. Lack of MMP-2 and MMP-9 affects eotaxin among other C-C chemokines, disrupts normal cell trafficking and favours the accumulation of eosinophils in parenchyma. With respect to the relationship of MMP-9 with TIMP-1, it has been reported that elevated MMP-9/TIMP-1 ratios were found in patients who did not need re-operation in relation to re-operated patients (16). This differed from previous results revealing that elevated MMP-9 expression in ECM in sinus mucosa at 1, 3, and 6 months after sinus surgery was linked to poor healing quality (4). A decrease in MMP-9 expression could be induced by incubation of the ECRS cultures with 6 ng/ml IL-13 in comparison to untreated controls in the present study. But these alterations in MMP-9 expression were insignificant compared to the alterations of the total protein concentrations. Deficiencies in MMP-9 expression are associated with reduced inflammatory cell apoptosis and a prolonged inflamatory response in allergen-induced airway inflammation (10, 11). Thus elevated IL-13 production by eosinophils in sinus mucosa might shift inflammatory reactions toward chronic inflammations. The reason for these seemingly incongruent results is probably the alterations of MMP functions in different disease phases.

In conclusion, the MMPs seem to play an important role in the remodelling of the ECM in CRS and changes in the expression of MMPs in the sinus mucosa could trigger the damage and dissociation of the epithelial barrier, thus contributing to alterations in mucosal permeability and pseudocyst formations. No direct correlations of MMP expression with IL-5 and -13, were demonstrated in this study.

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