

Original article

Relationship between matrix metalloproteinases MMP-2, MMP-9, tissue inhibitor of matrix metalloproteinases-1 and IL-5, IL-8 in nasal polyps

Background: Nasal polyps (NP), a subgroup of chronic rhinosinusitis, are characterized by interleukin 5 (IL-5) mediated infiltration of eosinophils in sinus mucosa, leading to pseudostratified ciliated columnar epithelium, thickening of the epithelial basement membrane and tissue edema. Matrix metalloproteinases (MMP) constitute a large group of Zn^{2+} dependent endopeptidases with the ability to degrade extracellular matrix and are possibly responsible for the development of tissue edema in chronic sinusitis.

Objective: The aim of this study was to determine the expression of MMP-2, MMP-9 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) mRNA and to locate the distribution of MMP-2, MMP-9 and TIMP-1 by immunohistochemistry in ethmoid sinus mucosa in NP. Furthermore the correlation between IL-5 or IL-8 and MMP-2, MMP-9 or TIMP-1 is examined.

Methods: Nasal polyps of 33 patients and 18 specimens of inferior turbinate mucosa were examined by real time RT-PCR for MMP-2, MMP-9, TIMP-1, IL-5 and IL-8 mRNA expression. Immunohistochemical labeling for MMP-2, MMP-9 and TIMP-1 was performed.

Results: Differences between both locations were detectable for MMP-9 ($P < 0.001$) and IL-5 ($P = 0.003$) but not for MMP-2 ($P = 0.278$), TIMP-1 ($P = 0.515$) and IL-8 ($P = 0.386$). Correlation was detected only between TIMP-1 and IL-5 ($r = 0.422$, $P = 0.014$). Cytoplasmic staining of MMP-2 was present in the apical part of the ciliated cells, submucosal glands and in smooth muscle cells. Matrix metalloproteinase-9 was expressed in surface epithelium, in seromucous glands and in polymorphonuclear cells.

Conclusions: Expression of MMP-9 and IL-5 mRNA are associated with NP. The correlation between IL-5 and TIMP-1 indicates the role of TIMP-1 in maintaining the homeostasis in NP.

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Nasal polyposis is considered a subgroup of chronic rhinosinusitis (1). Sinus mucosa in nasal polyps (NP) are characterized by massive stromal edema, infiltration with inflammatory cells like eosinophils, lymphocytes and plasma cells, alterations of the overlying epithelium and, in some cases, hyperplasia of submucosal seromucous glands (2). The factors leading to the morphological aspects of NP, such as massive infiltration with inflammatory cells, alteration of the respiratory epithelium and remodeling of the extracellular components, are poorly understood. Several cytokines and chemokines have been detected in increased levels in chronic sinusitis and nasal

polyps (3–9). Until now the link between elevated cytokine and chemokine levels and the development of edema and alterations of the extracellular matrix are subject to discussion. Possible factors for vascular permeability are vascular endothelial growth factor secreted by mast cells (10) and deposition of toxic mediators, such as eosinophil cationic protein and major basic protein, by degranulation of activated eosinophils that damage the epithelium (11). Recently, matrix metalloproteinases (MMP) were found to play a major role in normal physiologic tissue remodeling, inflammation and tumor spread. Matrix metalloproteinases are a group of Zn^{2+} -dependent endopeptidases, with more than 20 known members, that are able to degrade nearly every component of the extracellular matrix. Active forms of MMP-2 and MMP-9 share the ability to cleave type IV

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; NP, nasal polyp; IFT, inferior turbinate; IL, interleukin.

collagen. The extracellular activity of MMPs is regulated by tissue inhibitors of matrix metalloproteinases (TIMPs).

The role of MMPs and TIMPs in lung diseases is established (12). In transgenic animal studies elevated levels of MMP-2 and MMP-9 are associated with defects in bronchial architecture (13). Obviously, eosinophils are a source of MMP-9 in airway inflammation in chronic asthma (12). Although interleukin (IL)-5 is the predominant cytokine in NP and eosinophils are known as the source for MMP-9 direct stimulation of blood eosinophils with IL-5 did not lead to enhanced MMP-9 production (14).

Elevated levels of MMP-2 (15, 16), MMP-7 (17) and MMP-9 were found in NP (17, 18). Furthermore, extent of MMP-9 was found to predict healing after sinus surgery (19). Large amounts of TIMP-1 and TIMP-2 have been detected in nasal mucosa of patients suffering from allergic rhinitis as well as low levels of MMP-2 and MMP-9, but there was no significant difference in healthy control subjects (20). Elevated protein levels of TIMP-1 were detected in chronic sinusitis but not in NP (17).

Several cytokines are known to affect the production of MMP-2, MMP-9 and TIMP-1. In airway epithelium, IL-8 seems to have the potential of an initial activator of MMP-9 during airway epithelial regeneration (21). Interleukin-8 furthermore possesses the capability to induce MMP-2 and MMP-9 production in endothelial cells (22). In a study of relationship between MMP-1 and TIMP-1 enzymes and the TH1/TH2 cytokine network, by examination of blood in healthy donors, correlation was found between IL-5 and IL-8 serum levels and amount of TIMP-1 (23).

The capability of MMP-2 and MMP-9 to remodel the extracellular matrix, the association of these two endopeptidases in upper airway remodeling in asthma and the association with inflammation of the upper airways motivated us to investigate the possible role of these MMPs in the development of the morphological changes seen in NP. The description of influence of cytokines in production of MMP-2, MMP-9 and TIMP-1 led us to prove if there is a correlation between the extent of expression of MMP-2, MMP-9 or TIMP-1 and IL-5 or IL-8 in NP.

Methods

Subjects

The study group consisted of 33 patients (11 female, 22 male, median age 51 years, range 31–79) who underwent endonasal sinus surgery at the Department of Otorhinolaryngology, University of RWTH Aachen, Germany. All patients had clinical signs of chronic sinusitis (facial pain, congestion, rhinorrhea, etc.) for more than 3 months that had not improved after at least two courses of medical treatment including antibiotics and topical steroids for at least 14 days. All patients had nasal polyps confirmed by nasal endoscopy and signs of chronic sinusitis and nasal polyps in computed tomographic scans. Antibiotics and topical steroids were

discontinued 2 weeks prior to surgery. Acute infections were excluded by physical and laboratory examination. Fourteen (42%) patients of the nasal polyp group had a history of allergy confirmed by a positive skin test. In eight cases, seasonal allergy to grass pollen was found, while we detected perennial allergies in four cases to house mite dust. Two cases were allergic to both. Symptoms of allergy were reported by five patients at the time of operation. None of the patients with nasal polyps reported of intolerance for non-steroidal anti-inflammatory drugs. Four patients (12%) suffered from asthma. During endonasal microscopic sinus surgery from each single patient nasal polyps were obtained from the anterior ethmoid. Mucosa specimens of the inferior turbinate (IFT) from 18 patients (10 female, 8 male, median age 40 years, range 24–72) suffering from nonallergic vasomotor rhinitis, undergoing septal surgery without history, and clinical and radiological signs of chronic sinusitis and nasal polyps served as controls. The study protocol was approved of by the local ethics committee and informed consent was obtained from each patient.

Tissue preparation

Nasal polyps or IFTs were divided into two parts. One part was immediately flash frozen in liquid nitrogen for RNA extraction, the second part was fixed in buffered formalin and embedded in paraffin for conventional histopathological examination and immunohistochemistry.

RNA extraction and cDNA synthesis

Twenty to 30 mg of tissue samples were flash frozen in liquid nitrogen and homogenized using sterile pestles. Total RNA was prepared according to the manufacturer's instruction (Rneasy; Qiagen, Hilden, Germany). After a digestion step with RNase-free DNase (Roche Diagnostics, Mannheim, Germany) at 37°C for 1 h the total amount of RNA was measured with a spectrophotometer at 260 nm. One milligram of total RNA was reversely transcribed using a first strand cDNA synthesis kit for RT-PCR according to the manufacturer's protocol (Roche Diagnostics).

Quantitative real-time RT-PCR

Real-time RT-PCR on the LightCycler was performed using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). A total volume of 20 µl consisting of 1 µl of template (or water as negative control), 10 pmol of each primer, 2 µl of SYBR Green Fast Start reaction mix, 4 mM concentration of MgCl₂ for IL-5, IL-8, MMP-9 and TIMP-1 (3 mM for β-actin and MMP-2 primer set) and sterile water were prepared. Real-time PCR was performed in glass capillaries with an initial denaturation step of 10 min at 95°C followed by 40 cycles of 5 s at 95°C for denaturation, 5 s annealing temperature to 60°C for MMP-2, MMP-9 and TIMP-1 (IL-5 62°C and IL-8 66°C) followed by an extension step of 18 s at 72°C. At the end of each cycle, the fluorescence emitted by SYBR Green was measured after an additional interval of 2 s at 2°C below the specific melting temperature of the resulting product. After completion of the cycle process, the samples were subjected to a temperature ramp from 60 to 95°C at a rate of 0.2°C/s with continuous fluorescence monitoring for melting curve analysis. Primer sets were chosen from the literature for IL-5 and IL-8 (5), MMP-2 and MMP-9 (24) and TIMP-1 (25). Subsequently, the samples were separated in 1% agarose gels and stained with ethidiumbromide. For each PCR-product, a single narrow peak was obtained by melting curve analysis at the expected temperatures and

only a single band of the predicted size was observed after agarose gel electrophoresis, indicating specific amplification without significant byproducts. The specificity of the PCR was further confirmed by automated sequence analysis during the establishment of PCR conditions. All samples were measured at least twice.

Quantification of cDNA by real-time RT-PCR

The PCR method chosen quantifies PCR product during the log phase of the reaction. A calibration curve was generated automatically by a fourfold dilution series of one template ranging from 1 to 0.001 μ l of template solution. Samples were quantified accordingly by calculating the threshold cycle at which the fluorescent signal reaches an arbitrary but defined threshold value within the early exponential phase of the reaction (LightCycler[®] analysis software, version 3.39; Roche Diagnostics, Mannheim, Germany). For data analysis, the second-derivative method offered by the analysis software was chosen. The samples were standardized for the housekeeping gene β -actin. The relative expression of the examined cytokine was calculated (concentration cytokine/concentration β -actin) $\times$ 100.

Immunohistochemistry of MMP-2, MMP-9 and TIMP-1

Immunohistochemical analysis was performed on 4- μ m thick paraffin sections. Paraffin section of all samples were rehydrated and subjected to antigen retrieval by microwave treatment over three cycles of 750 W for 5 min in citrate buffer solution (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate buffer solution containing 0.025% Tween 20. Sections were subsequently incubated in blocking serum (Dako, Hamburg, Germany) for 3 h at 37°C, followed by the mouse monoclonal IgG1 kappa antibodies against both latent and activated forms of MMP-2, MMP-9 or against TIMP-1 (Oncogene, San Diego, CA, USA) at a dilution of 1 : 50 for 1 h at room temperature, and detected with the peroxidase LSAB kit (Dako). Slides were developed using a 3-amino-9-ethylcarbazole chromogen substrate. Breast cancer samples known to produce MMP-2, MMP-9 and TIMP-1 served as positive controls. Negative controls were obtained by omitting the primary antibody and replacing it with an irrelevant antibody of similar isotype.

Statistical analysis

The statistical analysis was performed with SPSS 13.0 for Windows (SPSS, Munich, Germany). The nonparametric Mann-Whitney *U*-test was used for comparison of relative MMP and TIMP expression between the two different groups. The Spearman correlation analysis was applied to evaluate the statistical significance between the two values. *P*-values of < 0.05 were considered as significant.

Results

Immunohistochemistry of MMP-2, MMP-9 and TIMP-1

Cytoplasmic staining of MMP-2 was observed in surface epithelium, glands and connective tissue. Matrix metalloproteinase-2 positivity was present in the apical part of the ciliated cells (Fig. 1A). Submucosal glands and smooth muscle cells of vessels were marked MMP-2-positive as well (Fig. 1B).

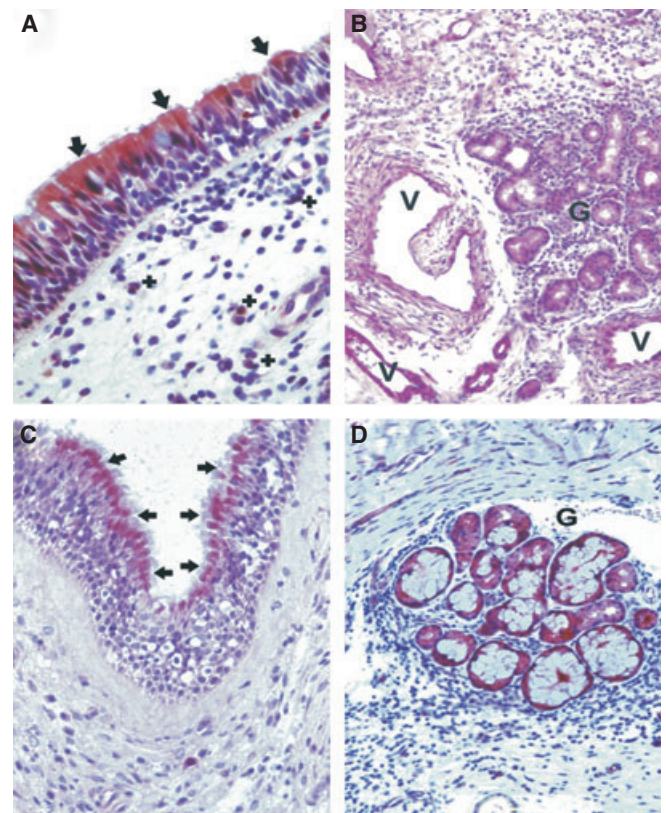


Figure 1. Representative matrix metalloproteinase (MMP)-2 (A) and MMP-9 (C) staining of surface epithelium (arrows) within nasal polyp and MMP-2 (B) and MMP-9 (D) staining of submucosal seromucous glands (G) and vessels (V) in nasal polyp (magnification: 200-fold).

Surface epithelium was also stained MMP-9-positive as well as polymorphonuclear cells (Fig. 1C). Intense staining of MMP-9 was detectable in seromucous glands (Fig. 1D).

Positive TIMP-1 staining was found in the basal layer of the respiratory epithelium. Ciliated cells were marked TIMP-1 positive but less intensely than MMP-2 and MMP-9. In contrast, submucosal glands showed various staining of TIMP-1 from light to intense (Fig. 2).

Relative mRNA Expression of IL-5, IL-8, MMP-2, MMP-9 and TIMP-1

Expression of IL-5 mRNA was found in three of 18 samples of IFT (17%) in low extent (mean = 0.015 ± 0.052), whereas IL-8 expression was detected in high concentration 5.23 ± 5.2 in nearly all samples (17 of 18). Relative expression of IL-5 mRNA in NP varied between 0 and 3.17 (mean = 0.31 ± 0.67) and was found in 20 of 33 (61%) samples. There was a significant difference between expression of IL-5 in IFT and NP (*P* = 0.003) (Fig. 3). Interleukin-8 mRNA was detected in high amount (mean = 3.68 ± 3.44) in nearly all NP

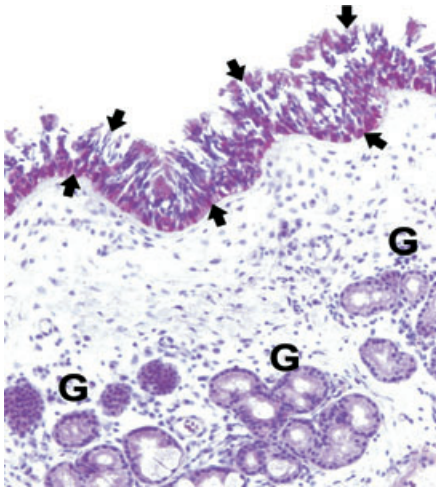


Figure 2. Staining with tissue inhibitor of matrix metalloproteinase-1 in surface epithelium (arrows) and submucosal seromucous glands (G) in nasal polyp (magnification: 100-fold).

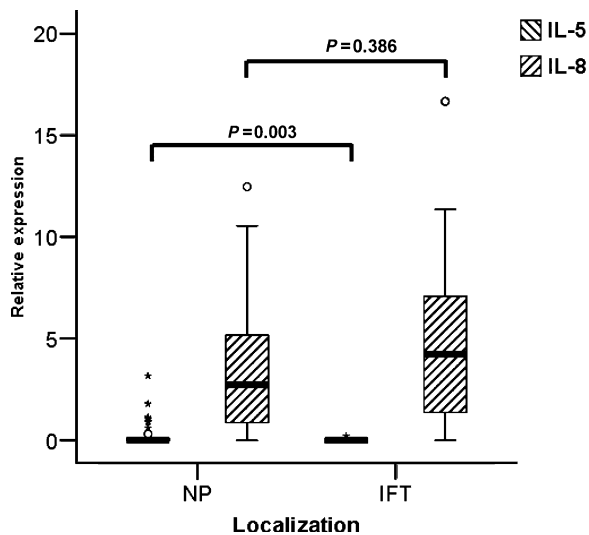


Figure 3. Comparison of relative expression of interleukin (IL)-5 and IL-8 between nasal polyp (NP) ($n = 33$) and inferior turbinate (IFT) ($n = 18$).

(32 of 33), but there was no difference between IFT and NP ($P = 0.386$) (Fig. 3).

In 17 of 18 samples of IFT (94%) MMP-2 was detected by real-time RT-PCR. The relative expression varied between 0 and 6.2 (mean = 1.75 ± 1.97). In all samples of NP MMP-2 mRNA was detected. Relative expression varied between 0.26 and 7.8 (mean = 2.21 ± 2.19). Statistical analysis showed no significant difference between relative expression of MMP-2 mRNA in IFT and NP ($P = 0.278$) (Fig. 4).

Matrix metalloproteinase-9 mRNA was detected in all samples of NP and in 16 of 18 specimens (89%) of IFT. Mean relative expression in NP was 0.96 ± 1.28 (range:

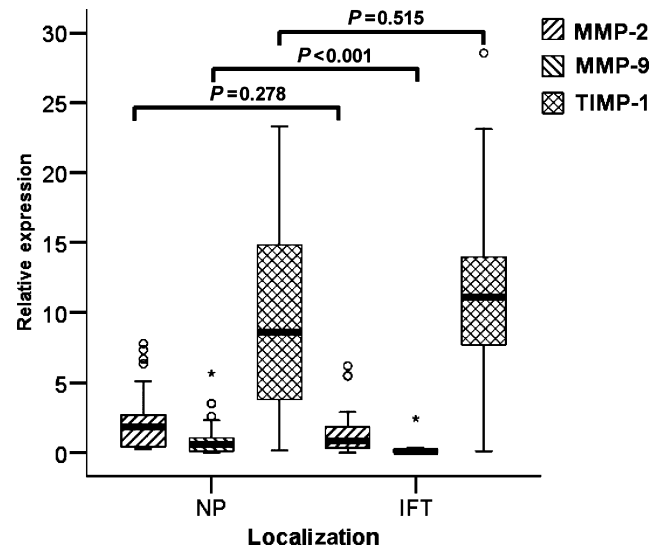


Figure 4. Comparison of relative expression of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) between nasal polyps (NP) ($n = 33$) and inferior turbinate (IFT) ($n = 18$).

0.21–5.66) and 0.25 ± 0.56 (0–2.44) in IFT. The difference in relative expression was statistically significant ($P < 0.001$) (Fig. 4).

Tissue inhibitor of matrix metalloproteinase-1 expression was detectable in all examined tissue samples with a mean relative amount of 9.85 ± 6.42 and range from 0.16 to 23.3 in NP and mean of 11.63 ± 7.18 and range from 0.11 to 28.53 in IFT. There was no difference in the relative expression of TIMP-1 ($P = 0.515$) between the two groups (Fig. 4). Comparing the expression of MMP-2, -9, TIMP-1, IL-5 and IL-8 in nasal polyps between atopic individuals ($n = 14$) and nonatopic individuals ($n = 19$) revealed no significant difference between the two groups of patients ($P > 0.05$).

Correlation between IL-5, IL-8 and MMP-2, MMP-9, TIMP-1

In IFT no significant correlation was found between extent of MMP-2, MMP-9 and TIMP-1 and IL-5 or IL-8 mRNA expression (Table 1).

IN NP TIMP-1 expression was positively correlated to IL-5 mRNA amount ($r = 0.422$, $P = 0.014$) (Fig. 5).

Discussion

This study compares the relative mRNA expression of MMP-2, MMP-9 and TIMP-1 in patients with nasal polyps and in the IFT of patients undergoing septal surgery without history, clinical and radiological signs of chronic sinusitis. The distribution of MMP-2, MMP-9 and TIMP-1 in nasal polyps is demonstrated by immunohistochemistry using specific antibodies. Furthermore,

Table 1. Correlation between relative expression of interleukin (IL)-5 or IL-8 and matrix metalloproteinase (MMP)-2, MMP-9 or tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in nasal polyps (NP) and inferior turbinate (IFT)

	NP			IFT		
	MMP-2	MMP-9	TIMP-1	MMP-2	MMP-9	TIMP-1
IL-5	$r = 0.094, P = 0.602$	$r = -0.239, P = 0.18$	$r = 0.605, P < 0.001$	$r = -0.116, P = 0.647$	$r = -0.124, P = 0.624$	$r = -0.325, P = 0.188$
IL-8	$r = 0.283, P = 0.111$	$r = -0.133, P = 0.459$	$r = 0.231, P = 0.195$	$r = -0.406, P = 0.094$	$r = -0.112, P = 0.658$	$r = -0.158, P = 0.532$

Significant results are given in bold.

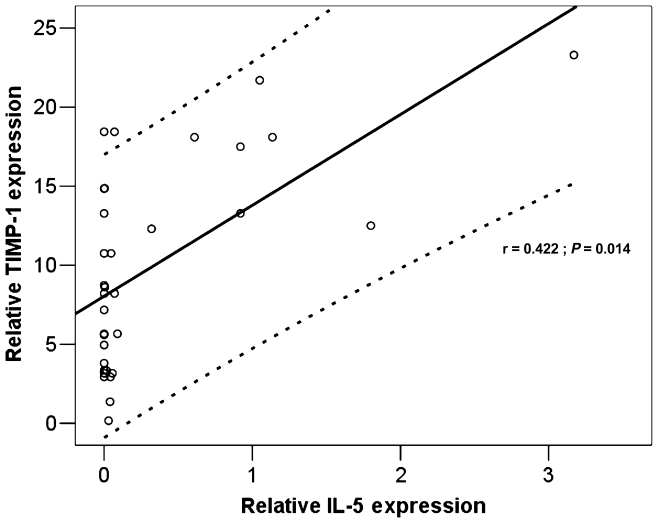


Figure 5. Correlation between relative expression of interleukin (IL)-5 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in nasal polyps ($n = 33$). Scattered lines mark 95% confidence interval.

the correlation of MMP-2-, MMP-9-, TIMP-1-expression with IL-8 and IL-5 was analyzed.

The relevance of MMP-2 in NP is discussed controversial in literature. Matrix metalloproteinase-2 is known to be produced by activated fibroblasts and its role in tissue repair and remodeling within the respiratory epithelium has been proven *in vitro* (26). Surface epithelium injured by inflammation might be a potential source for MMP-2. This hypothesis is supported by our immunohistochemical results as respiratory epithelium expresses MMP-2. Submucosal glands were also marked MMP-2 positive. These results are consistent with previous studies (16, 18). The presence of MMP-2 in NP was demonstrated in several studies (15, 16, 18) but only Bhandari et al. (16) detected higher MMP-2 mRNA expression in NP in subjects with nasal polyps and mucopurulent discharge than in IFT. Our result presents an equal amount of MMP-2 mRNA in NP and IFT and confirms the results of others measuring MMP-2 protein content by immunohistochemistry and zymography (18). The acute and possibly additional bacterial infection in the subjects included in the study mentioned above (16) may have led to divergent results. In our group of patients acute infection was treated with antibiotics and operation

was performed at the earliest 2 weeks after successful treatment.

Elevated expression of MMP-9 mRNA in NP compared with IFT confirms the result of previous studies (17–19). Only one study examined the mRNA amount of nasal polyps in subjects suffering of chronic rhinosinusitis with nasal polyps and mucopurulent nasal discharge came out with a different result (16).

The expression of the counterregulatory TIMP-1 shows no difference between NP and IFT in our study. This result confirms the presence of TIMP-1 in NP as in previous studies (15, 17) and equal amount of TIMP-1 protein in nasal polyps and IFT (17).

Expression of IL-5 mRNA was higher in NP than in IFT. This result underlines several previous studies pointing out the key role of eosinophils and IL-5 in nasal polyps (3, 4, 8, 9). In contrast, IL-8 mRNA was detectable in NP and IFT to a comparable extent. Although IL-8 is present in NP, it seems not to be specific for NP (4, 9) and is more often associated with acute or chronic inflammation of sinus mucosa (5, 27).

Interleukin-5 and MMP-9 mRNA are present in a higher extent in NP than in IFT, but we were unable to detect a correlation between expression of IL-5 and MMP-9 in NP. In previous studies on peripheral blood eosinophils in healthy subjects and patients with asthma, adding IL-5 did not lead to elevated production of MMP-9 (14). Another experiment demonstrated that only simultaneous stimulation of eosinophils with IL-5 and platelet-activating factor leads to an enhanced production of MMP-9 and elevated rate of eosinophil migration through basement membrane (28).

No correlation was also detected between MMP-2 and IL-5, but between IL-5 and TIMP-1 in NP. Tissue inhibitor of matrix metalloproteinase-1 mRNA was not significantly increased in NP compared with IFT, but a correlation was only detected between IL-5 in NP and not in IFT (Table 1). Besides the function of a specific inhibitor for MMP-9, TIMP-1 is shown to have growth-promoting activity in several cell types (29). Imbalance between MMPs and TIMPs may lead to delayed wound healing (30). Excess of TIMP-1 is supposed to be responsible for airway fibrosis in asthma (31). Interleukin-5 is not known to effect the production of TIMP-1 directly. Tissue inhibitor of matrix metalloproteinase-1 expression is regulated by IL-6 (32). Inflammatory cells including eosinophils are known as a source for IL-6.

Interleukin-6 has been detected in chronic sinusitis and chronic sinusitis with nasal polyps (5, 33, 34). We were not able to indicate a pathway of TIMP-1 expression in NP in this study, but the finding suggests complex interaction between inflammatory cells, respiratory epithelium and endothelial cells to maintain the balance of extracellular matrix remodeling in nasal polyps.

No correlation was found between IL-8 and either with MMP-2 or MMP-9 or with TIMP-1. An *in vivo* humanized airway xenograft model in nude mice, simultaneously examined the expression of IL-8 and MMP-9. IL-8 expression was elevated in the initial stages of airway epithelial regeneration followed by elevated levels of MMP-9 (21). In this model IL-8 served as an initial activator of MMP-9 in airway epithelium. Interleukin-8 was also described to stimulate neutrophils to release stored MMP-9 (35). Interleukin-8 detectable in NP (6, 9) but in higher extent after sinus surgery (6). In postoperative nasal secretions, elevated level of IL-8, extent of neutrophils (6) and MMP-9 (19) are signs of repair

processes that have taken place in sinus epithelium after injury. Our lack of a finding of a direct correlation between IL-8 and MMP-9 in NP indicates that in NP MMP-9 production through recruitment of neutrophils is not a relevant pathway for the formation of nasal polyps. There was no difference in expression of MMP, TIMP and interleukin in nasal polyps between patients with allergy and patients without allergy in our study. Nasal provocation with allergen is known to induce release of MMP-9 during the late-phase inflammatory response (36). The absence of allergic symptoms at the time of operation in the majority (9 of 14) of the patients with known allergy may have led to this result.

In conclusion, our study is consistent with previous studies reporting the key role of IL-5 and MMP-9 in NP. The correlation between IL-5 and TIMP-1 in NP underline the role of TIMP-1 in extracellular matrix remodeling in NP. The missing correlation between IL-8 and MMP-9 indicates a different mechanism in MMP-9 regulation in NP from that of repair of injured sinus epithelium.

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