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Distribution of matrix metalloproteinases MMP-1, MMP-2, MMP-8 and tissue inhibitor of matrix metalloproteinases-2 in nasal polyposis and chronic rhinosinusitis

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Summary. Nasal polyposis (NP), a chronic inflammatory disease of the upper airway, is a subgroup of chronic rhinosinusitis (CRS). Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are considered to play important roles in the pathogenesis of nasal polyposis. The aim of the current study was to evaluate and compare the levels of MMP-1, MMP-2, MMP-8 and TIMP-2 in NP and CRS with normal nasal mucosa by using immunohistochemistry. Twenty-five patients with NP and fifteen patients with CRS underwent endoscopic sinus surgery. Diseased mucosal samples were obtained from ethmoidal sinuses. Control nasal mucosa (n=10) was obtained from inferior nasal turbinate. Immunohistochemistry for MMP-1, MMP-2, MMP-8 and TIMP-2 was performed. The expression of MMP-1, MMP-2 and MMP-8 significantly increased in NP and CRS compared with control ($p<0.05$). The distribution of TIMP-2 was higher in CRS than control and NP respectively ($p<0.05$). MMP-1 immunoreactivity was distributed in the extracellular matrix whereas MMP-2, MMP-8 and TIMP-2 immunostaining was present in the epithelium, submucosal glands, vascular endothelium and inflammatory cells in CRS and NP. We suggest that differences in histological features between CRS and NP might be related to the expression of MMP-1, MMP-2, MMP-8 and their tissue inhibitor-2.

Key words: Matrix metalloproteinases-1, Matrix metalloproteinases-2, Matrix metalloproteinases-8, Tissue inhibitor of matrix metalloproteinases-2, Nasal polyposis

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Introduction

Nasal polyposis is a chronic inflammatory disease of paranasal sinuses which is an important health problem because of its recurrence, although both medical and surgical treatment are available (Kakoi and Hiraide, 1987). Sinus mucosa in NP are characterized by massive stromal edema, infiltration with inflammatory cells like eosinophils, mostly predominant infiltrative cells, lymphocytes and plasma cells, alterations of the overlying epithelium and, in some cases, hyperplasia of submucosal seromucous glands (Berger et al., 2002). Inflammatory cells release various cytokines and growth factors that are likely to regulate other features of nasal polyposis, such as fibroblast proliferation, extracellular matrix production, and epithelial cell proliferation and differentiation (Berger et al., 2002).

Several studies indicated that the balance between the matrix metalloproteinases (MMPs) and their regulators controlled the remodeling and repair of airway extracellular matrix (ECM) (Chen et al., 2007). The MMPs are a family of zinc-dependent endopeptidases that are collectively capable of degrading almost all components of the ECM (Birkedal-Hansen et al., 1993). They are also involved in various pathologic processes, such as inflammation, degenerative diseases and tumor spread (Birkedal-Hansen et al., 1993; Kostamo et al., 2005). Among the MMPs, the expression of MMP-2, MMP-7 and MMP-9 has been investigated in patients with chronic sinus diseases (Watelet et al., 2004; Chen et al., 2007; Can et al., 2008; Li et al., 2010). The authors demonstrated that diseased mucosa with or without NP exhibited elevated levels of MMP-2, 7 and 9 compared with control mucosa. However, little is known about the expression and the localization of MMP-1 and MMP-8 in chronic sinus diseases. Liu et al., (2001)

showed the relationship between MMP-1 expression in nasal polyp fibroblast and nasal polyp formation (Liu et al., 2001). Additionally, no change was observed in MMP-1 expression in patients with allergic rhinitis (Shaida et al., 2001).

Collagenase-2 (MMP-8), a member of an interstitial collagenase subfamily of MMPs, is a pivotal initiator of collagen breakdown at the sites of inflammation. MMP-8 was earlier regarded only as a neutrophil collagenase, but recent studies have shown that mesenchymal cells can also express MMP-8 (Hanemaaijer et al., 1997). Prikk et al. (2002) found in asthmatic patients strong MMP-8 immunoreactivity in bronchial epithelial cells (Prikk et al., 2002). Furthermore, MMP-8 has been shown to exhibit unexpected anti-inflammatory properties (Owen et al., 2004). Although several studies have focused on the expression of MMP-2, MMP-7, MMP-9 and the tissue inhibitors of metalloproteinases 1 (TIMP1) in patients with nasal polyposis, no study has investigated the differences in the localization of MMP-1, MMP-8 and TIMP-2 in patients with CRS and nasal polyps by using immunohistochemistry. Therefore, the goal of the current study was to assess the distribution of MMP-1, MMP-2, MMP-8 and TIMP-2 in CRS with or without nasal polyps. Therefore, we analyzed the expression of MMP-1, MMP-2, MMP-8 and TIMP-2 in normal and diseased nasal sinuses using immunohistochemistry.

Material and methods

Patients

Patients were selected at the Department of Otorhinolaryngology of Gaziosmanpasa University Hospital, Tokat, Turkey. Inferior turbinate samples from patients without sinus disease undergoing septoplasty or septorhinoplasty were collected as controls (controls, n=10; median age, 27 years; range, 18-45 years, 4 female, 6 male). Samples from patients with chronic rhinosinusitis (CRS, n=15, median age, 42 years; range, 34-78 years; 8 female, 7 male) and nasal polyposis (NP, n=25; median age, 46 years; range, 34-75 years; 15 female, 10 male) were obtained during functional endoscopic sinus surgery procedures. For CRS tissue samples originated from the ethmoidal sinuses. None of the control subjects or patients with CRS had a history of asthma, allergic rhinitis or aspirin sensitivity. In the NP group, 4 patients had a history of asthma, 2 patients had allergic rhinitis confirmed by positive skin prick test, and 1 patient had aspirin sensitivity. The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy, and computed tomography of the paranasal sinuses. Patients with nonallergic rhinitis with and without eosinophilia and vasomotor rhinitis were also excluded. None of patients had used oral corticosteroids for at least 2 months or topical application for at least one month before surgery. The study was approved by the local ethical committee of the Gaziosmanpasa University, Tokat, Turkey. Informed consent was obtained from each patient and control subject before collecting material.

Immunohistochemistry

The procedure for immunohistochemistry has been described elsewhere (Kayisli et al., 2006; Sati et al., 2009). 50 samples from control, diseased mucosa (CRS) and NP groups were used for immunohistochemical analysis. Tissues were fixed in 10% formalin and embedded in paraffin. Serial sections at 5 μm thickness were collected on poly-L-lysine coated slides (Sigma, St. Louis, MO, USA) and incubated overnight at 56°C. Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Sections were then treated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 5 min twice and left to cool for 20 min. After three washes in phosphate buffered saline (PBS), endogenous peroxidase activity was quenched by 3% hydrogen peroxide in PBS for 20 min and again washed three times in PBS. Sections were then incubated in a blocking serum (ScyTek Laboratories, USA) for 10 min in order to block non-specific binding. Subsequently, sections were incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: rabbit polyclonal MMP-1 (1:200, GeneTex, Irvine, USA), rabbit polyclonal MMP-2 (1:200, GeneTex, Irvine, USA), rabbit polyclonal MMP-8 (1:100, Abnova, Heidelberg, Germany), rabbit polyclonal TIMP-2 (1:50, Abnova, Heidelberg, Germany). After washing with PBS at room temperature, sequential incubations with biotinylated polyvalent antibodies (ScyTek Laboratories, USA) and peroxidase-labeled streptavidin (ScyTek Laboratories, Utah, USA) were performed. Immunohistochemistry was performed using a horseradish peroxidase-labeled streptavidin biotin (SensiTek HRP) kit (ScyTek Laboratories, Utah, USA) according to the manufacturer's instructions. Bound peroxidase was developed with 3-amino-9-ethylcarbazol (AEC) (ScyTek Laboratories, USA) chromogen and sections were counterstained with Mayer's hematoxylin (ScyTek Laboratories, Utah, USA) and mounted with Permount (Fisher Chemicals, Springfield, NJ, USA) on glass slides. For controls, sections were treated with the normal rabbit serum, depending on the primary antibody used, which was diluted to the same final protein concentration as the primary antibody. All samples for each individual antibody were exposed to the same protocol and were stained using the same incubation periods. Photomicrographs were taken with a Leica microscope (Leica DM2500, Nussloch, Germany).

HSCORE analyses

The evaluation of the immunohistochemical labelling was performed using HSCORE analyses (Kayisli et al., 2006). The intensity of MMP-1, MMP-2,

MMP-8 and TIMP-2 immunoreactivities were semi-quantitatively evaluated using the following intensity categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or distinct staining), and 3+ (intense staining). For each tissue, an HSCORE value was derived by calculating the sum of the percentages of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining, using the Formula HSCORE: $\sum Pi(i+1)$, where i represents the intensity scores and Pi is the corresponding percentage of the cells. In each slide, five randomly selected areas were evaluated under a light microscope (40x magnification), and the percentage of the cells for each intensity within these areas was determined at different times by two investigators who were not informed about type and source of the tissues. The average score of both observers was used.

Statistical analysis

Since the data from the HSCORE were not normally distributed, pairwise multiple comparisons were analyzed with non-parametric ANOVA on ranks (Dunn's Method) followed by Kruskal–Wallis test. Statistical calculations were performed using SigmaStat for Windows, version 3.5 (Jandel Scientific Corp., San Rafael, CA). Statistical significance was defined as $p<0.05$.

Results

Histological features of nasal tissues

Modifications of epithelial morphologic features and massive stromal edema were frequent in NP samples. We found predominantly pseudostratified ciliated epithelium in 18 cases, squamous metaplasia in 3 cases, and secretory hyperplasia in 4 cases. As expected, inflammatory cells like eosinophils, macrophages and lymphocytes were more numerous in NP samples and nasal mucosa from patients, but were rare in nasal mucosa from controls.

Immunohistochemistry of MMP-1, MMP-2, MMP-8 and TIMP-2

Immunohistochemical staining was used to determine the presence of MMP-1, MMP-2, MMP-8 and TIMP-2 in healthy and diseased sinonasal mucosal tissue. Representative sections of controls, CRS and NP are shown in Fig. 1.

MMP-1 was mainly localizated in the ECM of normal and diseased nasal mucosa (Fig. 1 a-c). Weak to moderate immunoreactivity for MMP-1 was observed in control mucosa (Fig. 1a) whereas strong immunoreactivity was seen in CRS and NP (Fig. 1b,c). Additionally, surface epithelium, mucosal and submucosal glands revealed very weak immunoreactivity for MMP-1 in the control mucosa, although some cells in the epithelium and glands revealed strong MMP-1 immunoreactivity in CRS and NP (Fig. 2a). Some, but not all inflammatory cells were stained for MMP-1 in CRS and NP (Figs. 1b-c, 2a). According to HSCORE analysis, the number and the intensity of the MMP-1 positive cells showed a significant increase in CRS and NP ($p=0.02$; $p=0.001$, respectively) (Fig. 3, Table 1).

In control group, MMP-2 staining was detected not only in the subepithelial region, but also in the epithelium (Fig. 1d). No immunolabelling was observed in the sections where the negative control antibody was used instead of MMP-2 primary antibody (Fig. 1d, inset). In CRS and NP, MMP-2 immunostaining was observed in the ciliated cells of epithelium, submucosal glands and inflammatory cells (Figs. 1e-f, 2b). As regards the intensity of MMP-2, there was a significant increase in CRS and NP ($p=0.03$; $p=0.01$, respectively) (Fig. 3, Table 1). Some basal cells of the epithelium and the vascular endothelium were stained with MMP-8 in control group. Moreover, vascular endothelium, submucosal glands and inflammatory cells, some of which were eosinophils, were positively labeled for

Table 1. Summary of our results and similar findings from literature.

Authors	samples*	MMP-1	MMP-2	MMP-8	TIMP-2
Lechapt-Zalcman et al.,2001	NP	-	no difference	-	-
Liu et al.,2001	NP	↑	-	-	-
Kostamo et al.,2005	NP	-	no difference	↑	-
Bhandari et al.,2004	CRS, NP	-	↑, ↑	-	-
Can et al.,2008	CRS, NP	-	↑, ↑	-	-
Leonardi et al.,2007	NP	↑	-	-	-
Chen et al.,2007	NP	-	no difference	-	-
Li et al.,2010	CRS, NP	-	no difference	-	no difference
Our results	CRS, NP	↑, ↑	↑, ↑	↑, ↑	↑, no difference

*: Samples from CRS (chronic rhinosinusitis) and NP (nasal polyposis) were compared to control. ↑ indicates higher expression; ↓ indicates lower expression of MMPs and TIMPs in CRS and NP respectively; 'no difference' indicates identical findings to control.

MMP-8 in CRS and NP (Figs. 1i-h, 2c). MMP-8 HSCORE analysis showed a significant increase in CRS and NP ($p=0.04$; $p=0.001$, respectively) (Fig. 3, Table 1).

TIMP-2 displayed intense immunoreactivity in both control group and CRS without NP (Fig. 1j-k). Ciliated epithelial cells showed TIMP-2 positivity but more intensely than MMP-2 and MMP-8 staining. Similar to MMP-2 and MMP-8 staining, some but not all inflammatory cells were moderately stained with TIMP-2 in CRS and NP. TIMP-2 positive cells were more abundantly present in the epithelium and endothelial cells of blood vessels in CRS (Fig 1k). Additionally, submucosal glands showed various staining of TIMP-2 from moderate to intense (Fig. 2d). The HSCORE values

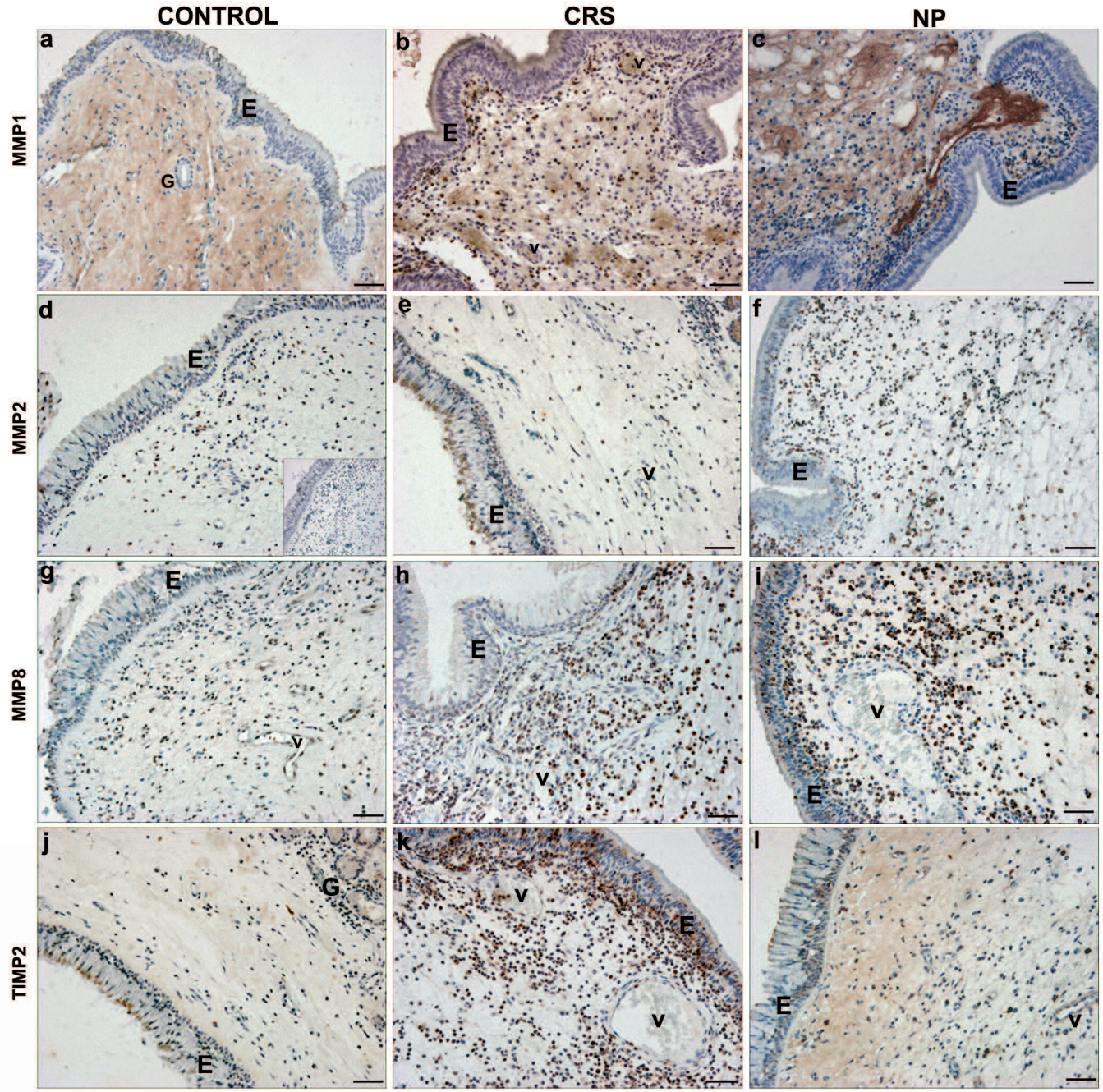

Fig. 1. Immunostaining for MMP-1 **(a-c)**, MMP-2 (**d-f**), MMP-8 (**g-i**), TIMP-2 (**j-l**) in control, CRS (chronic rhinosinusitis), NP (nasal polyposis). Epithelium (E), vascular endothelium (V), glands (G) are shown. Scale bar: 50 μm.

of TIMP-2 staining obtained from CRS were significantly higher than the control and NP (p=0.02; p=0.01, respectively) (Fig. 3, Table 1).

Discussion

The current study investigated the expression of MMP-1, MMP-2, MMP-8 and TIMP-2 in CRS, NP and control using immunohistochemistry. The expression of MMP-1, MMP-2 and MMP-8 was higher in NP than CRS and control respectively. Although the expression of MMP-1, MMP-2 and MMP-8 in NP was not significantly different from CRS, TIMP-2 expression significantly increased in CRS compared with control and NP. Similarly, other studies comparing protein expression of MMP-2 and MMP-8 in patients with NP versus control have found that MMP-8, but not MMP-2, expression was significantly increased in patients with NP compared with control (Kostamo et al., 2005) (Table 1). There is limited knowledge about the expression of MMP-8 in patients with NP. Kostamo et al. (2005) evaluated enhanced MMP-8 expression in chronic sinusitis by using Western blot analysis. They found increased expression of MMP-8 together with increased activation of mesenchymal type MMP-8, but not polymorphonuclear type MMP-8 in CRS. Most patients had allergic rhinitis or asthma and 2 of 13 patients had aspirin sensitivity in their study. For the first time we show, using immunohistochemistry, the localization and the expression of MMP-8 in patients with and without NP.

Our findings for the significantly increased expression of MMP-2 in patients with or without nasal polyps compared with controls are in accordance with the findings of others (Bhandari et al., 2004; Can et al., 2008) (Table 1). In contrast to these findings, Lechapt-

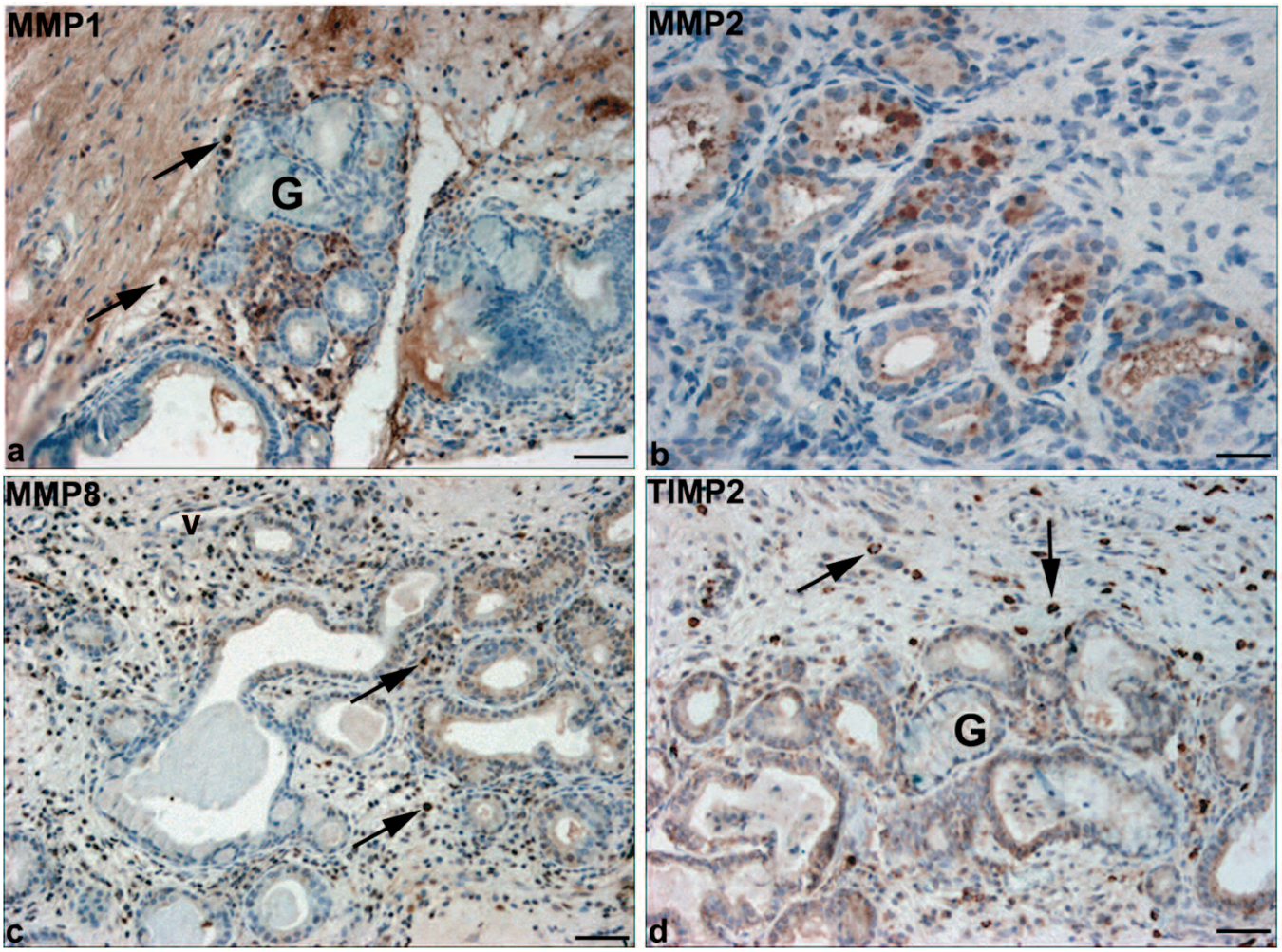

Fig. 2. Immunostaining of MMP-1 **(a)**, MMP-2 **(b)**, MMP-8 **(c)** and TIMP-2 **(d)** in the submucosal glands (G), vascular endothelium (V) and inflammatory cells (arrows) of nasal polyps. Scale bar: 50 μm.

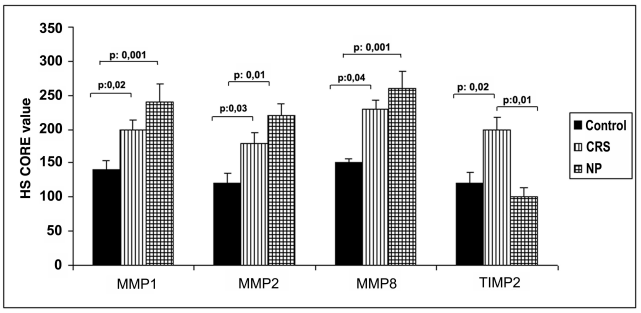

Fig. 3. Comparison of the HSCORE values of MMP-1, MMP-2, MMP-8 and TIMP-2 in control, CRS and NP groups. The data are represented as mean ± SEM.

Zalcman et al., (2001) evaluated both the active form and latent form of MMP-2 and MMP-9 with in situ zymography and immunohistochemistry in nasal polyposis and normal nasal mucosa. The active and latent form of MMP-9 demonstrated a significant increase in NP compared to normal nasal mucosa. However, both forms of MMP-2 did not demonstrate a difference in NP compared to normal nasal mucosa. Chen et al. (2007) obtained similar results indicating increased expression of MMP-9 but not MMP-2 in NP. Moreover, a recent study found no significant differences in MMP-2 concentration in Chinese patients with or without nasal polyps (Li et al., 2010) (Table 1). The authors showed that Chinese patients did not have asthma and aspirin sensitivity but had positive skin test (6 of 12 patients in CRS and 2 of 12 patients in NP).

We have also observed increased expression of MMP-1 in NP. In accordance with our finding Leonardi et al., (2007) also found increased expression of MMP-1 in nasal polyp with allergic rhinitis (Table 1). Moreover, Liu et al., (2001) investigated the induction of IL-1α and PGE2 on synthesis of MMP-1 and TIMP-1 in nasal polyp fibroblasts and nasal mucosal fibroblasts. They found an increased amount of MMP-1 mRNA in nasal polyp fibroblasts compared to nasal mucosal fibroblasts. However, the synthesis of TIMP-1 in both groups was similar. They suggested that MMP-1 might play a role in the development of nasal polyp.

In contrast to our finding showing the increased expression of MMP-1 in patients with NP and CRS, only one report demonstrated no upregulation of MMP-1 in the nasal mucosa of patients with allergic rhinitis (Shaida et al., 2001). In their study, not only MMP-1, but also other MMPs, showed no changes. Despite these discrepancies, it is more likely that differences in the expression of MMPs lead to specific changes in tissue remodeling in patients with or without NP.

One of the major control mechanisms of MMP synthesis and functions is provided by TIMPs, endogenous inhibitors of MMPs (Gueders et al., 2006). There is equilibrium between MMPs and TIMPs related to their expression, activation and inhibition to maintain constriction of ECM. Four different types of TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been identified in vertebrates. All of them show different affinity for different types of MMPs (Gueders et al., 2006). Many studies have focused on TIMP-1, a general inhibitor of MMPs (Watelet et al., 2004) in NP and CRS (Liu et al., 2001; Lee et al., 2003; Watelet et al., 2004; Kostamo et al., 2005; Leonardi et al., 2007; Can et al., 2008; Shin et al., 2009). TIMP-2 is able to inhibit both active and latent forms of MMP-2 and MMP-9. Also, TIMP-2 recruits activation of pro-MMP-2 (Strongin et al., 1995; Seiki et al., 2003). In our study, TIMP-2 immunoactivity significantly reduced in NP compared to CRS but increased in CRS compared to control group. In contrast to our findings, Li et al. (2010) found that TIMP-2 concentration did not show any differences in NP, CRS and control group in Chinese patients (Table 1).

The relevance of MMPs and TIMPs are controversial in literature according to our knowledge. The relationship between MMPs and their tissue inhibitors is thought to be critical in regulating tissue remodeling, which also applies to CRS and NP. In this study, MMP-1 was detected around cells in stroma, suggesting rapid ECM degradation. On the other hand, MMP-2 and MMP-8 immunpositivity was found in the epithelial, endothelial and inflammatory cells in CRS and NP. The presence of immunpositive inflammatory cells in ECM pointed to a direct role in the degradation of ECM. When they are activated, inflammatory cells respond and release MMPs, creating a pseudocyst formation and stromal edema, which are commonly observed in NP.

In contrast to MMPs, reduced immunoreactivity of TIMP-2 in NP was found in our study. Additionally, TIMP-2 is upregulated in CRS, modulating or preventing MMP activity. This may explain why a pseudocyst formation and massive stromal edema in CRS is not present. The occurrence of differences in the level of MMPs and TIMPs in NP and CRS has reflected by the typical ECM remodeling patterns in patients with and without nasal polyposis. In this regard, our study is also concordance with the finding of others (Watelet et al., 2004; Kostamo et al., 2005; Chen et al., 2007; Li et al., 2010).

In conclusion, our study reports that MMP-1, MMP-2, MMP-8 and TIMP-2 are expressed and more likely contribute to tissue remodeling in CRS and NP, with different distributions compared with controls. These differences might be the consequence of different patient profiles, histomorphologic features of tissues and co-existent diseases, including allergic rhinitis, asthma and aspirin sensitivity.

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