

Localization of Active Caspase-3 and Caspase-8 in Nasal Polyps and Nasal Hyperplasia in Consideration of Mast Cell Function: A Semiquantitatively Analysis

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ABSTRACT

Introduction: The pathogenesis of nasal polyposis and nasal hyperplasia is still unknown. The localization of caspases in nasal polyps and nasal hyperplasia of patients with and without allergic rhinitis was studied. **Methods:** Sections of human nasal polyps (n = 5) and hyperplastic nasal turbinates (5 with, 5 without allergy) were stained for active caspase-3 and caspase-8. Double immunofluorescence was used to evaluate colocalization of the caspases with Ki-MIP and tryptase. TUNEL was performed. **Results:** Active caspase-3 and caspase-8 were seen in nearly all nasal polyps and hyperplastic nasal turbinates. Active caspase-3 was predominantly localized in stromal cells, identified as mast cells. Caspase-8 was localized in mast cells with the pattern similar to active caspase-3 and additionally found in epithelial cells at the nasal and polyp surface and in epithelial cells of glands. **Conclusion:** Our results suggest that mast cell apoptosis may be involved in the pathological mechanisms which characterize and sustain chronic inflammatory disorders of the nasal mucosa with and without allergy.

Keywords: Nasal Polyposis; Caspase-3; Caspase-8; Allergic Rhinitis; Non-Allergic Rhinitis; Mast Cells; Nasal Pathology; Chronic Rhinosinusitis; Apoptosis; Nasal Hyperplasia

1. Introduction

Over the last decade chronic inflammatory nasal diseases have become more and more frequent and the rate of their occurrence continues to grow. This trend is not solely attributable to the incidence of allergic rhinitis. Nasal polyposis is the ultimate manifestation of chronic inflammation of the upper airway mucosa [1]. Though not fatal, nasal polyposis is a common nasal disease with a high rate of recurrence [2]. The pathogenesis of nasal polyposis is still enigmatic. Several investigations give evidence that the accumulation of inflammatory cells, especially mast cells and eosinophils and their increased amount of inflammatory mediators have enormous influence on polyp development [3-5]. Apoptosis, often defined as programmed cell death, is an essential mecha-

nism including several important physiological and pathological processes to eliminate unwanted cells during the development, to initiate remodelling of tissues and to regulate homeostasis of multicellular organisms [6,7]. An imbalance between apoptosis and survival is responsible for various diseases, like ischemic damage, neurodegenerative and autoimmune diseases or cancer [8-10]. A deregulation of this system also seems to play a central role in the pathogenesis of nasal polyposis [2,11,12]. Several investigations give evidence that the accumulation of infiltrating inflammatory cells in nasal polyposis is caused by a decrease in apoptosis [13-15]. Kowalski *et al.* (2002) found a strong positive correlation between the duration of nasal polyposis and the density of apoptotic cells in atopic patients [13]. In their investigation, TUNEL, a nick end-labelling technique to detect DNA strand breaks, and apoptotic morphology was used

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to identify apoptosis. Caspases are key players in programmed cell death. Depending on the nature of stimuli and cell type, two major caspase activation pathways have been described: The death receptor-mediated caspase-8-dependent extrinsic pathway and the mitochondrial-initiated intrinsic pathway [16-18]. Activated initiator caspases (caspase-8 or -9) start a downstream cascade of effector caspases, such as caspase-3, which cleaves various substrates and leads to the execution of cell death [19,20]. Nevertheless, this pathway is not a one-way mechanism, but to some point can be reversible. Results of the localization of caspases in healthy nasal mucosa are contradictory. Whereas some exclude the presence of caspases in healthy nasal tissue [21], recent studies describe caspase activity in normal mucosa as well as in nasal pathologies [22,23]. In order to understand better the role of caspases during the pathogenesis of chronic nasal diseases, we investigated the localization of caspase-3 and -8 in nasal polyps and in nasal mucosal hyperplasia of patients with and without nasal allergy.

2. Material and Methods

15 patients (7 females, 8 males, mean age 39.14 ± 17.99 , range 21 - 70 years) were included in the study. Nasal polyps were obtained from 5 patients (all males, mean age 54 ± 10.37 , range 41 - 68) undergoing elective polypectomy or conchotomy at the department for ENT of the Georg-August-University Hospital, Goettingen, Germany. The second group consisted of 10 patients (7 females, 3 males, mean age 41 ± 18.67 , range 21 - 70) with hyperplastic middle nasal turbinates (5 with and 5 without nasal allergy). Nasal allergy was defined by a positive personal history in allergic rhinitis symptoms and a positive skin prick test. None of the patients had an ongoing drug treatment. Nasal surgery was unrelated to the goal of this study. The study was approved by the local committee on human experimentation and patients gave their consent to participate. Tissue fixation, HE-staining and immunohistochemistry were done as described elsewhere [24] by using caspase-3-antibody (polyclonal rabbit-anti-human/mouse-caspase-3-active-antibody, R & D Systems, Wiesbaden, Germany), diluted 1:500 in TBS-BSA 1% for 2 hours and bridge-antibody (goat-anti-rabbit-immunoglobulin-antibody, DAKO A/S, Glostrup, Denmark), diluted 1:50 with TBS-BSA.

For localization of caspase-8 the indirect immunoperoxidase-method was applied using monoclonal mouse-anti-human-FLICE/caspase-8-antibody as first antibody (Beckman Coulter, Krefeld, Germany) over night in a dilution of 1:100 in TBS-BSA and a peroxidase conjugated rabbit-anti-mouse-immunoglobulin-antibody as second antibody (DAKO A/S, Glostrup, Denmark), incubated for 30 minutes, diluted 1:50 in TBS-BSA. DAB-

Tris-HCl-solution served as chromogen. Rinsing between all steps was performed in TBS-buffer for 10 minutes. Incubation of antibodies was done in a humid chamber at room temperature. The final steps included counterstaining with hemalaun (according to Mayer) and mounting with DPX (Fluka, Steinheim, Germany). Controls included omission of the primary antibodies and replacement by TBS/BSA 1% in every charge.

For double immunofluorescence staining paraffin sections were put on silanized glass slides, deparaffinised, treated with boiling citrat buffer 3 times for 5 minutes in a microwave, cooled down and rinsed with water and TBS. Endogenous peroxidase activity was blocked with 10% BSA. Anti-caspase-3-antibody- (diluted 1:100 in TBS) versus anti-caspase-8-antibody (diluted 1:50 in TBS) served as first antibody, incubated over night in a humid chamber. Sections were then incubated with LINK (goat-antimouse/rabbit, DAKO, A/S, Glostrup, Denmark) for 30 minutes. From this point all steps were carried out in darkness: Incubation with streptavidin FITC (DAKO A/S, Glostrup, Denmark, diluted 1:40 in TBS, marked with green fluorescence) for 60 minutes and incubation with an unmarked F(ab)-fragment (goat-anti-mouse, Dianova, Hamburg, Germany, diluted 1:50 in TBS) for further 30 minutes followed. To identify the origin of the caspase-positive free connective tissue cells, a primary antibody against mast cell tryptase (mouse-anti-human, DAKO, A/S, Glostrup, Denmark, diluted 1:100 in TBS) versus Ki-M1P (monoclonal, mouse-anti-human, Radzun *et al.* [25], diluted 1:500 in TBS) was then added. A Cy3-marked F(ab)-fragment (goat-anti-mouse, Dianova, Hamburg, Germany, diluted 1:25 in TBS, marked with red fluorescence) served for staining. Rinsing between all steps was done in TBS-buffer for 5 minutes. Fluorescent-mounting medium (DAKO, Carpinteria, CA, USA) was used. Qualitative assessment: The extent of caspase-positive free connective tissue cells was graded semiquantitatively with a light microscope "KF 2" (Zeiss, Oberkochen, Germany) as (++) if there was a high amount of caspase-3/-8-positive cells, (+) if there was only a small extent of caspase-3/-8-positive cells, and (-) if no caspase-3/-8-positive cells could be detected. In the epithelial cells at the nasal and polyp surface and in the epithelial cells of the glands the staining for caspase-3 and -8 was graded as (+) if a clear staining was detected, and as (-) if there was no clear staining for caspase-3/-8. In these epithelial cells the clear staining was separated over again in a clear granular staining (1), a fine, sporadic granular staining (2) and a homogeneous staining (3). TUNEL-staining: The TUNEL assay was conducted with the ApopTag Fluorescein In Situ Apoptosis detection kit by Intergen (cat. S7111, Billerica, MA, USA) according to manufacturer's instructions. Images of the labelled cells were taken with

a fluorescence inverted-microscope (Leica DFC 350 Fx, Bensheim, Germany, Software Qwin Image 4.0).

3. Results

Localization and quantitative evaluation of active caspase-3 and caspase-8 in nasal polyps and in hyperplastic nasal mucosa of non-allergic and allergic patients are summarized in **Tables 1-6**. In nasal polyps, active caspase-3 was detected in 4 of the 5 nasal polyps (see **Table 1**). It was mainly localized in free connective tissue cells (**Figure 1**), whereas a few free intraepithelial cells, positive for active caspase-3, presumably inflammatory cells, were present at the polyp surface. A colocalization for active caspase-3 and mast cell tryptase in free connective tissue cells (**Figure 2-4**) was seen. In contrast, no colocalization for active caspase-3 and Ki-M1P was detected. Caspase-8 was found in all 5 nasal polyps (see **Table 2**). Similar to caspase-3, caspase-8 was localized in free connective tissue cells (**Figure 5**) and in one case also in free cells within the epithelium at the polyp surface. It was additionally found in epithelial cells at the polyp surface itself and in epithelial cells of glands. In most of this tissue, a clear staining for caspase-8 was noted within epithelial cells at the polyp surface, whereas epithelial cells of glands showed a clear staining for caspase-8 only in one nasal polyp, basically in excretory ducts. A colocalization for caspase-8 and mast cell tryptase in free connective tissue cells of the nasal polyps (**Figure 6-8**) was seen. Similar to caspase-3, no colocalization for caspase-8 and Ki-M1P was apparent. In hyperplastic nasal mucosa of patients without nasal allergy, active caspase-3 was localized in free connective tissue cells in 4 of 5 hyperplastic nasal turbinates (see **Table 3**, **Figure 9**). By double immunofluorescence, a colocalization for active caspase-3 and mast cell tryptase in the free connective tissue cells was found, whereas no colocalization for active caspase-3 and Ki-M1P was detected. Caspase-8 was found in all specimens (see **Table 4**), and in free connective tissue cells (**Figure 10**). Four of them showed clear staining of epithelial cells at the nasal surface. 4 of 5 hyperplastic nasal turbinates showed caspase-8-positive free cells within the epithelium at the nasal surface. Epithelial cells of glands were stained irregularly (**Figure 10**, inlay down to the left). Double immunofluorescence staining for caspase-8 and mast cell tryptase showed a colocalization in the free connective tissue cells, but no colocalization for caspase-8 and Ki-M1P. In hyperplastic nasal turbinates of patients with nasal allergy, localization of active caspase-3 and caspase-8 corresponded to that of the tissue of patients without nasal allergy (see **Tables 5 and 6**, **Figures 11 and 12**); in one case this staining was predominantly localized in the excretory ducts (patient 12). TUNEL-staining revealed a clear and specific signal for a few positive cells per sec-

tion, recognizable in all samples scattered in the stroma, from both the hyperplastic nasal turbinates of patients

Table 1. Localization of caspase-3 active in nasal polyps.

Patient	1	2	3	4	5
Free connective tissue cells	++	-	++	+	++
Free cells in the epithelium at the polyp surface	-	-	+4	-	-
Epithelial cells at the polyp surface	-	-	-	-	-
Epithelial cells of the glands	-	-	-	-	-

Table 2. Localization of caspase-8 in nasal polyps.

Patient	1	2	3	4	5
Free connective tissue cells	++	+	++	++	++
Free cells in the epithelium at the polyp surface	-	-	-	-	+4
Epithelial cells at the polyp surface	+1	+1	+2	-	+3
Epithelial cells of the glands	+2	-	-	**	-

Table 3. Localization of caspase-3 active in hyperplastic nasal mucosa without nasal allergy.

Patient	6	7	8	9	10
Free connective tissue cells	++	++	++	-	++
Free cells in the epithelium at the polyp surface	-	-	-	-	-
Epithelial cells at the polyp surface	-	-	-	-	-
Epithelial cells of the glands	-	-	-	-	-

Table 4. Localization of caspase-8 in hyperplastic nasal mucosa without nasal allergy.

Patient	6	7	8	9	10
Free connective tissue cells	++	++	++	++	++
Free cells in the epithelium at the polyp surface	+4	+4	-	+4	+4
Epithelial cells at the polyp surface	+3	+3	+1	-	+3
Epithelial cells of the glands	+3	-	-	-	+3

Table 5. Localization of caspase-3 active in hyperplastic nasal mucosa with nasal allergy.

Patient	11	12	13	14	15
Free connective tissue cells	++	++	++	+	-
Free cells in the epithelium at the polyp surface	-	-	-	-	-
Epithelial cells at the polyp surface	-	-	-	-	-
Epithelial cells of the glands	-	-	-	-	-

with or without nasal allergy and the nasal polyps, as revealed by fluorescence microscopy (**Figure 13(A)-(E)**). Additionally, isolated positive cells for the TUNEL staining were also found within the epithelium in some sections (**Figure 13(C)**). Interestingly, TUNEL-positive signal was also revealed in the cytoplasm of some cells in the stroma (**Figure 13(E)**).

4. Discussion

The accumulation of inflammatory cells, especially mast cells, eosinophils and their inflammatory mediators seem to have a key role in the development of nasal polyps [26,27]. Caspase activation takes up an important posi-

Table 6. Localization of caspase-8 in hyperplastic nasal mucosa with nasal allergy.

Patient	11	12	13	14	15
Free connective tissue cells	++	++	++	++	++
Free cells in the epithelium at the polyp surface	-	+4	-	-	+4
Epithelial cells at the polyp surface	+1	-	+1	-	-
Epithelial cells of the glands	-	+3	+2/+3	-	-

Legend for free connective tissue cells: (++) high extent/amount of caspase-3/-8-positive cells; (+) small extent of caspase-3/-8-positive cells; (-) no clear staining for caspase-3/-8. Legend for free cells within the epithelium at the polyp and nasal surface, epithelial cells at the polyp and nasal surface and epithelial cells of the glands: (+) clear staining for caspase-3/-8; (-) no clear staining for caspase-3/-8; (*) no glandular structures in the tissue; 1) clear granular staining; 2) fine, sporadic granular staining; 3) homogeneous staining clearly visible; 4) few caspase-3/-8-positive free cells in the epithelium at the polyp and nasal surface.

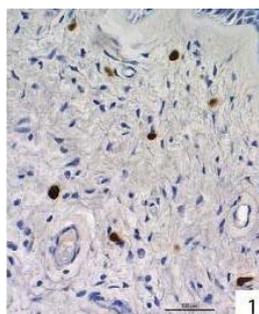


Figure 1. Active caspase-3 was seen in 4 of the 5 nasal polyps. Immunohistochemical localization of active caspase-3 in free cells in the connective tissue of a nasal polyp.



Figure 2. Double immunofluorescence staining for active caspase-3 in a nasal polyp.



Figure 3. Double immunofluorescence staining for mast cell tryptase in a nasal polyp.



Figure 4. Double immunofluorescence staining: colocalization for active caspase-3 and mast cell tryptase in a nasal polyp. No colocalization for active caspase-3 and Ki-MIP was detected.

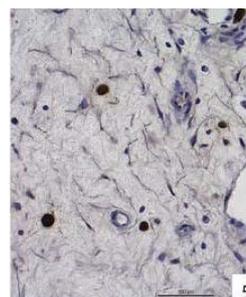


Figure 5. Caspases-8 was detected in all 5 polyps. Immunohistochemical localization of caspase-8 in free cells in the connective tissue of a nasal polyp. Caspase-8 was also found in free cells within the epithelium at the surface of one polyp, in epithelial cells at the polyp surface and in epithelial cells of glands (the latter 3 localizations are not shown).

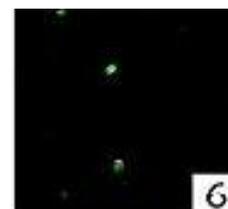


Figure 6. Double immunofluorescence staining for caspase-8 in a nasal polyp.

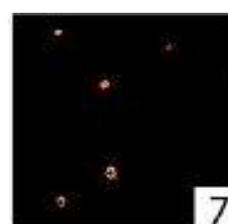


Figure 7. Double immunofluorescence staining for mast cell tryptase in a nasal polyp.



Figure 8. Double immunofluorescence staining: colocalization for caspase-8 and mast cell tryptase in a nasal polyp. No colocalization for caspase-8 and Ki-M1P was detected.

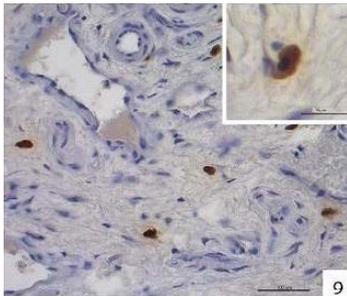


Figure 9. Immunohistochemical localization of active caspase-3 in free cells in the connective tissue of hyperplastic nasal mucosa (without allergic rhinitis). Inlay up to the right: active caspase-3-positive cell.

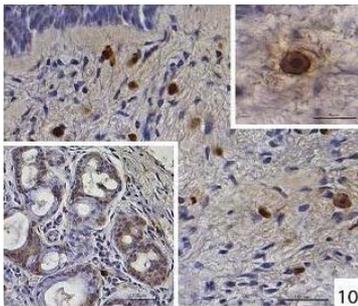


Figure 10. Immunohistochemical localization of caspase-8 in free cells in the connective tissue of hyperplastic nasal mucosa (without allergic rhinitis). Inlay up to the right: caspase-8-positive cell. Inlay down to the left: localization of caspase-8 in the glands in hyperplastic nasal mucosa (without allergic rhinitis), diffuse irregular staining.

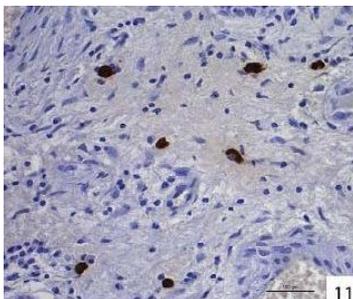


Figure 11. Immunohistochemical localization of active caspase-3 in free cells in the connective tissue of hyperplastic nasal mucosa (with allergic rhinitis).

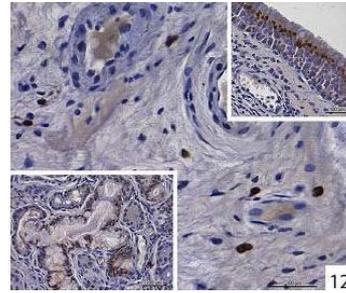


Figure 12. Immunohistochemical localization of caspase-8 in free cells in the connective tissue of hyperplastic nasal mucosa (with allergic rhinitis). Inlay up to the right: localization of caspase-8 in the surface epithelium in hyperplastic nasal mucosa (with allergic rhinitis), granular staining. Inlay down to the left: Localization of caspase-8 in the glands in hyperplastic nasal mucosa (with allergic rhinitis), granular staining.

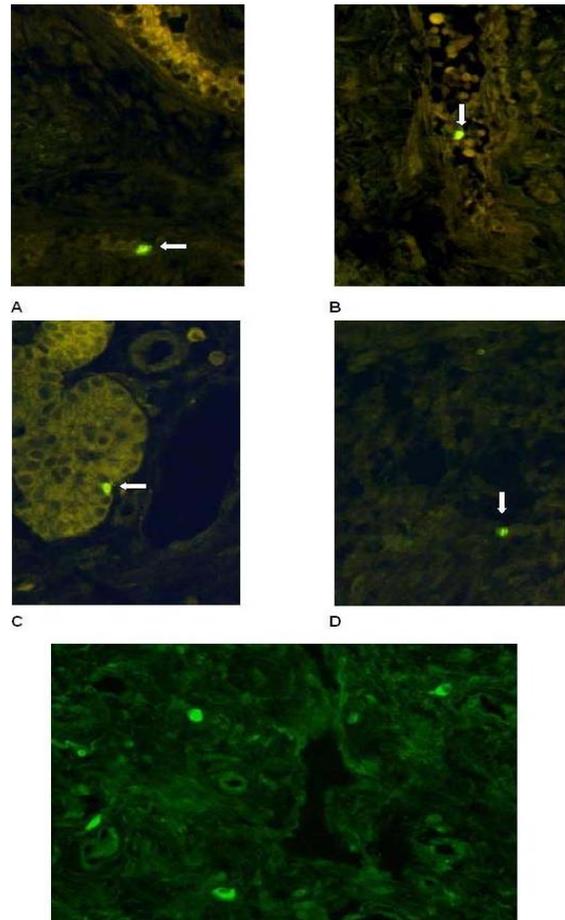


Figure 13. (A) + (B): TUNEL-staining. Hyperplastic nasal mucosa (without allergic rhinitis, (A)) and nasal polyp (B) with scattered positive cells in the stroma (arrow). (C) + (D): TUNEL-staining. Hyperplastic nasal mucosa (with allergic rhinitis) with scattered positive cells in the stroma (D). Note the isolated positive cells localized additionally within the epithelium ((C), arrow). (E) TUNEL-staining. Nasal polyp. Positive signals were detected in the cytoplasm of some cells in the stroma (arrow).

tion in the execution of apoptosis [7]. Nevertheless, little is known about the cellular localization of caspases and their involvement in chronic inflammatory nasal diseases, entities in which apoptosis could be reduced or even increased. Our study reveals the localization of caspase-3 and caspase-8 in human nasal polyps and hyperplastic nasal mucosa in patients with and without nasal allergy. Lately, rare caspase-3 activity has been shown in nasal mucosa of the lower nasal turbinates in healthy controls, whereas no or isolated caspase-3 activity in allergic rhinitis and defined activity in the endothelium and in the lamina muscularis of subepithelial vessels in polyposis nasi was detected [22]. Cho *et al.* (2008) found no significant differences in the level of expression of caspase-3 between normal mucosa and nasal polyps [23]. We found presence of both active caspase-3 and caspase-8 within nearly all nasal polyps and hyperplastic nasal turbinates primarily localized in scattered cells in the stroma without substantial differences in all groups. These results implicate similar involvement of active caspase-3 and of caspase-8 in the cellular pathological mechanisms of nasal polyposis and hyperplasia of nasal mucosa, regardless of the coincidence of nasal allergy. Double immunofluorescence labelling revealed localization of both caspases in mast cells, whereas localization in macrophages could be excluded. Caspase-3 is classically activated as one of the common effectors in the apoptotic cascade [19,20]. Its presence in mast cells of nasal polyps and hyperplastic nasal mucosa points out its participation in chronic nasal disorders. This is corroborated by the fact that scattered TUNEL-positive cells were found in connective tissue, indicating occurrence of apoptotic cell death in the stroma of the hyperplastic nasal turbinates. The fact that caspase-positive staining was widespread in the cytoplasm of mast cells points out a slow and long lasting activation of caspase-3 before DNA degradation is finally triggered [28]. Mast cells are known to undergo apoptosis, both in a caspase-dependent and a caspase-independent manner [29]. However, they are exceptionally long-living cells (up to months). Their number is, at least under normal *in vivo* conditions, relatively constant, suggesting that they are normally not programmed for spontaneous apoptosis [30]. Therefore, it is likely that elevated activation of caspases-3 in mast cells may be part of the mechanisms which underline pathogenesis of nasal polyps and mucosal hyperplasia. The presence of TUNEL-positive cells in the stroma underlines that cell death occurs in this cellular population. Apoptosis has been described as an essential host defence mechanism in order to prevent spreading of infections and to eliminate unwanted cells during tumour growth [31,32]. Some groups have reported caspase-3 and -8-mediated apoptosis in human mast cells *in vitro* or in mastocytoma and in various inflammatory and fibrotic

diseases as liver fibrosis, Crohn's and chronic graft-versus-host-disease [31,33,34]. Based on their *in-vitro* investigations of human mast cell lines, Berent-Moaz *et al.* (2006) proposed that the extrinsic apoptotic pathway, mediated by the death receptor TRAIL-R (tumor necrosis factor-related apoptosis-inducing ligand) and resulting in caspase-3 activation, might be a mechanism of regulating mast cell survival *in vivo* and, potentially, for down-regulating or resolving mast cell hyperplasia in diseases [35]. According to this, caspase detection in mast cells of nasal polyps and hyperplastic nasal mucosa, *in vivo* as shown in this work, could reduce and resolve or even prevent mast cell hyperplasia. Another reason for the presence of active caspase-3 and -8 in mast cells in chronic nasal diseases may not primarily be related to ongoing cell death, but may indicate the involvement in maturation and processing of pro inflammatory cytokines, as has been reported for other caspases [36]. Mast cells are a cellular source of IL-16, a potential chemotaxin for CD⁺ T-cells and CD4⁺ eosinophils [37]. Eosinophils in turn are known to be important effectors in polyposis nasi and allergic rhinitis, so that caspase-3 may play a mature role in the inflammatory process of chronic nasal disorders. Whereas active caspase-3 was nearly exclusively stromal, caspase-8 was additionally found almost consistently in the cytoplasm of epithelial cells at the nasal and polyp surface and in glands. The fact that just some TUNEL-positivity was seen in epithelia, and that staining of active caspase-3 in these compartments was limited to intraepithelial inflammatory cells, suggests that no massive death is occurring in epithelial cells. The caspase-8 antibody used in our study cannot distinguish between the active and inactive form of caspase-8. We propose that caspase-8 might be constitutionally expressed in the nasal epithelium and just waits for a proper signal to be activated. Whether or not this is normally the case in all epithelia or if it is a hallmark of chronically damaged tissues, remains unclear to this point and requires further investigation. Interestingly, in some cells, TUNEL signal was clearly evident in the cytoplasm.

As it has been described for olfactory sensory neurons [38], phagocytic cells might engulf dying cells which contain fragmented DNA. Phagocytic cells are negative for caspase-3 and -8, and therefore are not expected to be TUNEL-positive. The presence of phagocytosed DNA-fragments in their cytoplasm might explain the cytoplasm positivity to TUNEL. It might indicate that for example cell death in the nasal stroma contributes to these pathologies.

5. Conclusion

We showed expression of active caspase-3 and caspase-8 in stroma cells, which have been identified as mast cells. This together with the presence of TUNEL suggests that

ongoing cell death of mast cells characterize and sustain chronic inflammatory disorders of the nasal mucosa.

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