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The pathogenesis of chronic inflammation and malignant transformation in the human upper airways: the role of beta-defensins, eNOS, cell proliferation and apoptosis

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Summary. The surrounding environment contains plenty of pathogens, which represent a danger of infection. The simplest way for the pathological microorganism to enter the organism is the upper airways. Inflammation of the upper airways is among the most common and frequent diseases. This category includes nasal polyposis and chronic tonsillitis. In many cases it is associated with disorders in relation to the immune response. An inflammatory infiltration of mononuclears, eosinophils, plasma and mast cells can be found in the histological structure of the polypous as well as tonsillar mucosa.

One aim of this study was to determine the expression of beta-defensins and various proteins, with a possible potential role in relation to the rise and development of those changes. Another aim was to determine the relationship between the inflammatory and malignant processes in the tonsils.

The samples of nasal polyps were obtained during clinically indicated endonasal surgery from patients diagnosed with nasal polyposis (n=50). The samples of tonsils were collected during surgery from patients suffering from chronic tonsillitis (n=11) or tonsillar carcinoma (n=17). Immunohistochemical procedures for the detection of human beta-defensin 1, 2, 3 (HBD-1, 2, 3), Ki- 67, endothelial nitric oxide synthase (eNOS) and cleaved caspase 3 were performed on cryostate and paraffin sections.

It was proven that HBD are secreted in fairly large amounts in cases of chronic inflammation. Their secretion during the malignant transformation is limited. This is a very probable fact that plays a role in malignant transformation in tonsillar tissue. The crucial role in the development of chronic inflammation, and maybe that of malignant transformation, is played by eNOS and its product NO molecule. eNOS and the NO molecule are involved in cell cycle regulation, in the apoptotic processes and cell proliferation, as well as in the angiogenesis and vasculogenesis. Our result confirmed that eNOS is presented in the tissues of the upper airways in both chronic inflammation and carcinomatous processes. Ki-67 and cleaved caspase 3 were used as markers of cell proliferation and apoptosis.

Key words: Tonsillitis chronica, Tonsillar carcinoma, Nasal polyposis, Defensins, Apoptosis, Proliferation

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Introduction

The simplest way for the pathological microorganism to enter the organism is through the upper airways. Inflammation of the upper airways is among the most common and frequent diseases. This category includes nasal polyposis and chronic tonsillitis. In many cases it is associated with disorders in relation to the immune response. There are some mechanisms to protect the organism against infection; in the upper airways there is a lot of lymphatic tissue in the mucous layer. In that association it can be claimed that the nasopharyngeal tonsil, pharyngeal tonsils, lingual tonsil and palatine tonsils - these accumulations of lymphatic tissue - belong to the so-called Waldayer's ring (Hellings et al., 2000). In addition to tonsillar apparatus, there are a lot of cells participating in the immune system all over the nasal cavity, nasopharynx and oropharynx. The components of the mucosa produce different substances, which help to keep the mucosal surface clear of infection.

Included in these components is the family of peptides called defensins. These peptides function as endogenous antibiotics and are active against bacteria,

fungi and enveloped viruses (Lee et al., 2002). One group of those peptides, beta-defensins, is commonly produced by epithelial cells from the superficial as well as glandular epithelium, which can be found on mucosal lining, as well as in the epithelial cells of different organs (airways, digestive tract, reproductive tract) – the potential gateways for infection. These molecules are involved in the innate mucosal immunity (Diamond et al., 2001). Some authors have reported the incidence of HBD-1, 2, 3 in the nasal as well as the tonsillar epithelium. HBD-1, 2, 3 were present in both the clinically healthy and the pathological mucosa (Chae et al., 2001; Weise et al., 2002; Wang et al., 2004). HBD-2 was found to have a significantly higher expression in tonsils with chronic inflammation, in contrast to HBD-1, which was detected at corresponding levels in healthy and inflamed tonsils (Dunsche et al., 2001). HBD-2 was found in significantly higher levels in inflamed nasal mucosa, HBD-1 was present in approximately the same quantities in healthy and polyposis mucosa (Carothers et al., 2001). HBD-1 was not regularly found in tumorous tissue. On the contrary, HBD-2 was found in significantly higher levels in keratinocytes of squamosus cell carcinoma. From this it results that HBD-2 might play an important role in the origin of squamous cell carcinoma (Mizukawa et al., 2000; Sawaki et al., 2002; Yoshimoto et al., 2003). HBD-3 was also studied in the superficial epithelium of tonsils and nasal mucosa (Dunsche et al., 2002; Meyer et al., 2006).

There are known changes in cell proliferation and apoptosis during inflammation and malignant transformation. We have focused our efforts on studies regarding the following compounds: eNOS, Ki-67 and cleaved caspase-3. eNOS is involved in inflammation processes and carcinogenesis. It can modulate cancer related events (angiogenesis, apoptosis, cell cycle, invasion and metastases). It is a key inflammation mediator because it can regulate the expression of the proinflammatory molecules (e.c. cyclooxygenase-2) (Blais and Rivest, 2001). It was found that both mucosal and vascular tumor cells have shown dysregulation of eNOS and it has been proven that angiogenesis is a key step in solid tumor progression. eNOS is involved in both angiogenesis (the development of new blood vessels from existing vessels) and vasculogenesis (blood vessel formation de novo from progenitor cells) (Duda et al., 2004). Some authors have found that NO mediates the branching and longitudal expression of the blood vessels and that this process is predominantly mediated by eNOS. eNOS plays an essential role in endothelial cell proliferation and is a central mediator of several endothelium growth stimulators, such as VEGF (vascular endothelial growth factor) and PGE2 (prostaglandin E2) (Duda et al., 2004; Namkoong et al., 2005). In addition to tumor cell expression, eNOS is particularly expressed by vascular endothelial cells or surrounding stromal cells (Brouet et al., 2005). eNOS also has a function for apoptosis. The NO molecule can have either an antiapoptotic or proapoptotic effect; lower concentrations of NO have an antiapoptotic effect (Li and Wogan, 2005; Fukumura et al., 2006; Lancaster and Xie, 2006). Apoptosis was visualized by studying the apoptotic marker: cleaved caspase-3. Proliferation is also influenced by eNOS. The proliferation was studied in a squamous cell carcinoma of the oropharynx by detection of the marker for proliferation Ki-67 (Grabenbauer et al., 2000). It is a key molecule for endothelial cell activation and proliferation. The effect of the NO molecule depends on the specific cell type, the genetic background of the target and the NO concentration (Shang et al., 2006). It was found that eNOS and associated NO can increase the permeability of the tumor-blood barrier and therefore may play a role in tumor invasion (Jadeski et al. 2006; Ying et al., 2007).

Immunohistochemical detection was performed to establish the possible role of human beta defensin-1, 2, 3 (HBD-1, 2, 3) and various proteins (endothelial nitric oxide synthase (eNOS), Ki- 67 as a proliferative marker and cleaved caspase 3 as the apoptotic marker in the etiopathogenesis of nasal polyposis and chronic tonsillitis. It was the first aim of this study.

Another aim was to determine the relationship between the inflammatory and malignant processes in tonsils.

Material and methods

The samples of nasal polyps were obtained during clinically indicated endonasal surgery from patients with nasal polyposis (n=50). The tonsil samples were collected during surgery from patients suffering from chronic tonsillitis (n=11) or tonsillar carcinoma (n=17). For lightmicroscopy the samples were fixed in 4 % paraformaldehyde for imbedding to paraffin or snap frozen in liquid nitrogen and kept frozen at -80°C. Paraffin and cryostate sections were used for the detection of defensins (HBD-1, 2, 3), endothelial nitric oxide synthase (eNOS), Ki-67 and cleaved caspase 3 via the three-step immunoperoxidase methods. The detection on cryostat sections was carried out on 4% PFA in PBS-fixed frozen sections. The paraffin sections were deparaffined using xylen and 96% ethanol. The 2% fetal bovine serum (Biosera, UK) in PBS was used for 30 minutes to block non-specific binding of immunoglobulins. The next step in processing was incubation with primary antibody [Anti-Human-HBD-1 Rabbit Polyclonal Antibody (Alpha-Diagnostic, Inc., USA) 1:400, Anti-Human-HBD-2 Rabbit Antiserum (Peptide Institute, Inc., Japan) 1:200, Anti-Human-HBD-3 Rabbit Polyclonal Antibody (Orbigen, USA) 1:100, Anti-Human-NOS-3 Rabbit Polyclonal Antibody (eNOS) (Santa Cruz Biotechnology, USA) 1:100 and Anti-Human-Cleaved Caspase-3 Rabbit Monoclonal Antibody (Cell Signaling Technology, USA) 1:200, Anti-Human-Ki-67 Mouse Monoclonal Antibody (Immunotech, France) 1:100. This was performed for 60 minutes at room temperature. After washing in PBS, the sections were incubated with biotinylated Goat-Anti-

Rabbit IgG in PBS (Sigma-Aldrich, USA) 1:200 or Goat-Anti-Mouse IgG in PBS (Sigma-Aldrich, USA) 1:400 as the secondary antibody for another 30 minutes at room temperature. After repeated rinsing in PBS three times for another 5 minutes, the sections were incubated with Vectastain ABC Elite kit peroxidase (VECTOR lab., USA) diluted in PBS for ABC buffer (1:50) for 30 minutes at room temperature. Five minutes in PBS gently rinsed preparations were exposed to Diaminobenzidine (DAB) peroxidase substrate solution (DAKO Cytomation, Denmark) until brown staining appeared. The nuclei were counterstained with hematoxylin for 5 seconds.

The antigen retrieval was performed after deparaffination in TRIS buffer base (pH 9.5) in a microwave oven (1 minute 560W and 5 minutes 240W). Any possible activity of endogenous peroxidase was blocked by 70% methanol and 1% hydrogen peroxide for 10 minutes. The above mentioned procedures were performed on cryostate sections with the exception of antigen retrieval.

Results

HBD-1 was detected in the stratified squamous epithelium, especially in the so-called keratine pearls in the carcinoma tissue. Chronic tonsillitis was characterized by findings of HBD-1 in the covering stratified squamous epithelial cell cytoplasm in the most superficial layers, as well as in stratum spinosum. The

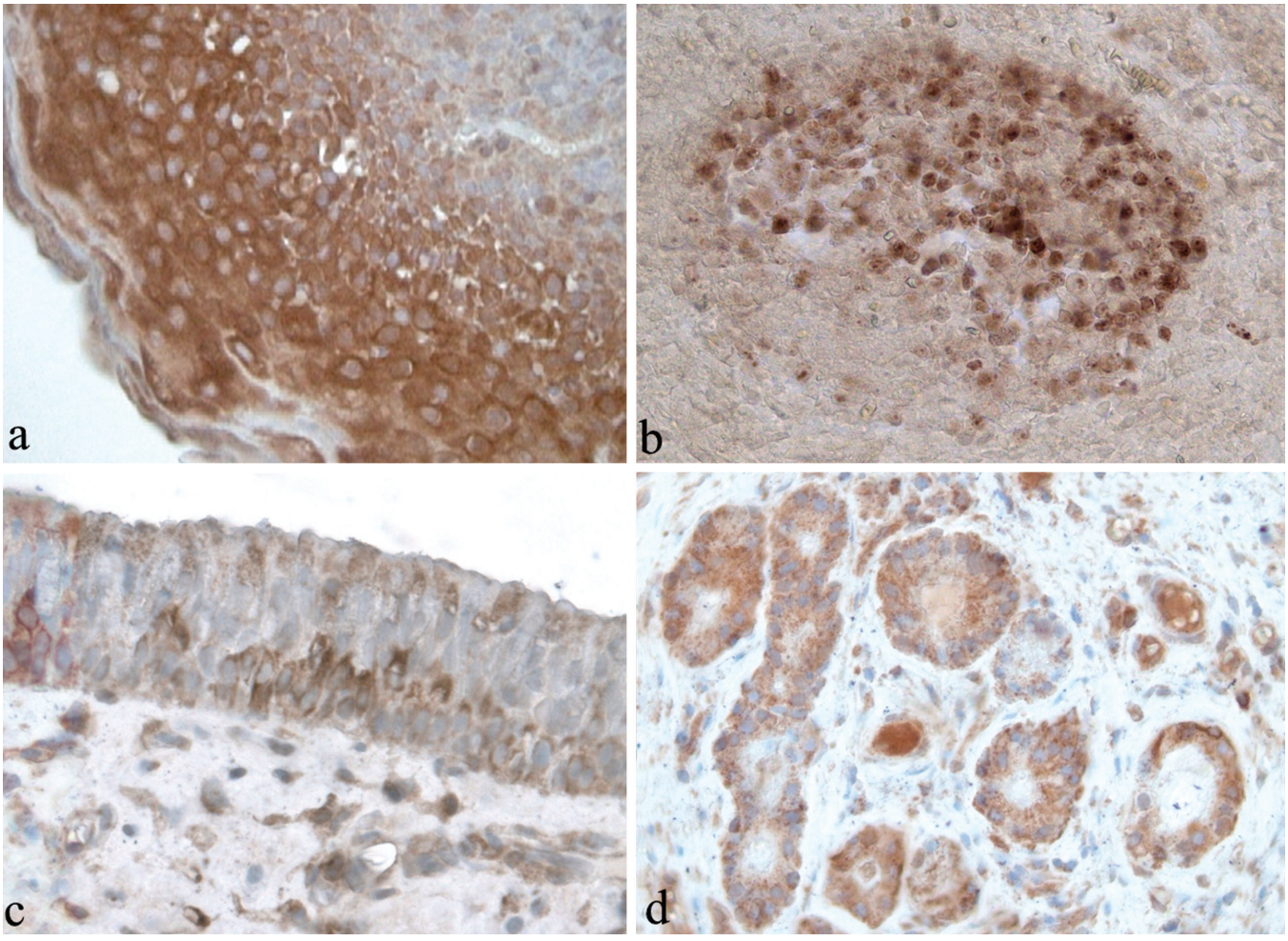

Fig. 1. The samples of chronically inflamed tonsils **(a, b)** and nasal polyps **(c, d)**. Immunoperoxidase reaction (DAB). **a.** The positivity of HBD-2 in the cell cytoplasm of the superficial stratified squamous epithelium in chronic tonsillar inflammation. Counterstained by Hematoxylin. Obj. magn. x 63. **b.** The positive reaction of Ki-67 in the lymphocyte cytoplasm in the germinative center of the tonsillar lymphatic follicles during chronic inflammation. Obj. magn. x 40. **c.** The detection of HBD-3 in the nasal polypous tissue. The positivity is present diffusely in the perinuclear cytoplasm of the epithelial cells; the granular pattern can be seen in the apical cytoplasmatic portion of these cells. The positivity was also demonstrated in some cells of the connective tissue. Counterstained by Hematoxylin. Obj. magn. x 63. **d.** The granular pattern of the positive reaction of HBD-3 in the mucous glandular cell cytoplasm in the nasal polyp. Counterstained by Hematoxylin. Obj. magn. x 63

reaction product was only detected in some of the samples. In the polypous tissue, the presence of HBD-1 was not regular; slight positivity was observed only in the apical portion of the cytoplasm in some cells in the pseudostratified epithelium. HBD-1 was also found in the cytoplasm of the secretory cells in the glandular epithelium.

HBD-2 was dispersed in the cell cytoplasm of the superficial stratified squamous epithelium (stratum spinosum) and in some macrophages passing through the epithelium of the carcinoma tissue, but only at low levels. This defensin was demonstrated in the tonsillar inflamed tissue, especially in the superficial squamous epithelium – in its apical portion and stratum spinosum (Fig. 1a), as well as in the marginal layers of the lymphatic follicles. In the pseudostratified epithelium of nasal polyps HBD-2 was regularly distributed in the apical cytoplasm of cells – a more or less granular pattern of reaction product distribution was found.

HBD-3 can be found again in the superficial epithelium of the tonsillar carcinoma tissue (stratum spinosum) but not in the keratine pearls (Fig. 2a). It was detected in high amounts mostly in the apical portion and stratum spinosum of the covering epithelium in the inflamed tonsils. The reaction product was also found in the cell cytoplasm of the marginal layer of lymphatic follicles. In the polypous tissue, HBD-3 was demonstrated diffusely in the perinuclear cytoplasm of the epithelial cells, a granular pattern was found in the apical cytoplasmatic portion of these cells, as well as in the mucous glandular cells. Positivity was also detected in some connective tissue cells, some macrophages in the polypous stroma and in the cytoplasm of the endothelial cells of small vessels (Fig. 1c,d).

eNOS was detected at high levels mostly in the keratine pearls in the superficial epithelium of the

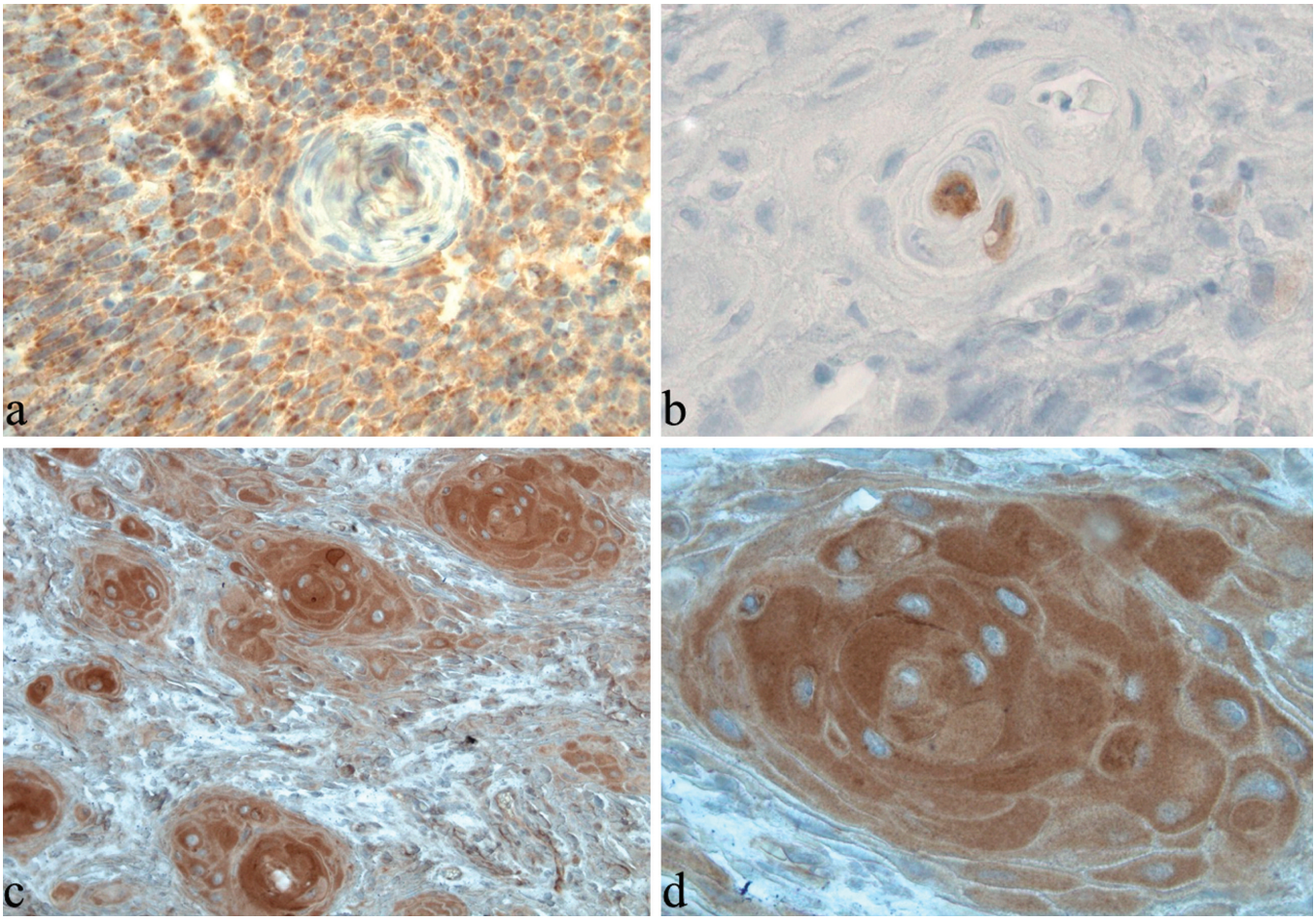

Fig. 2. The samples of tonsillar carcinoma. Immunoperoxidase reaction (DAB). Counterstained by Hematoxylin. **a.** The expression of HBD-3 in the cell cytoplasm of the stratified squamous epithelium. The keratin pearl does not show positivity. Obj. magn. x 63. **b.** The apoptosis is demonstrated by the detection of cleaved caspase 3 in the keratin pearl of the superficial epithelium. Obj. magn. x 63. **c.** The expression of eNOS in the keratine pearls. Obj. magn. x 20. **d.** The expression of eNOS in the keratine pearls. Obj. magn. x 63

carcinoma tissue (Fig. 2c,d). It was found in the cytoplasm of endothelial cells of the stromal vein in a very large amount. In the tonsillar inflamed tissue it was found at high levels in the cytoplasm of the covering epithelial cells and in some mononuclears in the tonsillar stroma. In high amounts it was detected in the germinative centers of the lymphatic follicles and in the endothelial cytoplasm of high endothelial veins. In the nasal polyps the eNOS was detected on the apical cilia of the covering pseudostratified columnar epithelium; high levels were also found in the cell cytoplasm of the high endothelial veins.

Ki-67, the proliferative marker, was detected in the nuclei of basal epithelial cells in the nasal mucosa of polyps. Fortunately, in one sample, Ki-67 was also found in the capillary endothelium. Ki-67 positive cells in the connective tissue of lamina propria were detected in variable numbers. Numerous positive cells were detected in the germinative centers of lymphatic follicles, as well as in the basal layers of the superficial squamous epithelium in chronic tonsillitis (Fig. 1b). Ki-67 was detected in the carcinomatous tissue, especially in the germinative centers of the lymphatic follicles in the lower levels in the keratine pearls of the superficial epithelium.

Cleaved caspase 3, the apoptotic marker, was detected only rarely in a few of the tissue samples examined. Apoptotic bodies were occasionally found in the epithelium and some apoptotic cells were present in the connective tissue of the lamina propria mucosae in polyps. In the chronically inflamed tonsils it was particularly found in the germinative centers of the lymphatic follicles. In tonsillar carcinoma some apoptotic bodies were detected in the keratine pearls of the superficial epithelium (Fig. 2b) and very rarely in the germinative centers of the lymphatic follicles.

Discussion

The preliminary results, obtained from the immunohistochemical investigation of some potential agents that can contribute to the development of nasal polyposis, chronic tonsillitis and their possible connection with tonsillar carcinoma, confirmed the complicated physiological and pathological pathways in the upper respiratory tract. Although HBD-1 is known as the defensin, which is mostly present in healthy tissues (Carothers et al., 2001; Dunsche et al., 2001), it can be found - according our findings - also in inflammatory and carcinoma tissue, although its levels are quite low. HBD-2 and HBD-3 synthesis can be induced only in certain inflammatory stimulations. Our findings demonstrated that they could also be detected in tonsillar carcinoma tissue. HBD-2 was found in large amounts in the inflamed samples but its presence in the carcinomatous tissue was very poor, nearly none in contrast to the literature results where HBD-2 was found in high levels in the superficial stratified squamous epithelium of tonsils with carcinoma (Mizukawa et al., 2000; Sawaki et al., 2002; Yoshimoto et al., 2003). Our results confirm that cancerogenesis can be facilitated by the absence of this defensin. HBD-3 was detected in the superficial stratified squamous epithelium of tonsils in higher levels than HBD-1 and 2, although these levels were much lower than those found in the inflamed tissues. This shows that during malignant transformation the production of this defensin is limited. eNOS is present in the covering epithelial cell cytoplasm in both chronic inflamed and carcinomatous tonsils. Typical localization of reaction product in the cytoplasm of the endothelial cells of capillaries and small vessels confirmed accelerated angiogenesis. eNOS and its product NO are involved in processes such as angiogenesis and vasculogenesis, apoptosis and proliferation, cell cycle regulation and the regulation of vascular permeability (Blais and Rivest, 2001). Our results confirmed that eNOS is presented in the tissues during chronic inflammation as well as during carcinomatous processes. This was detected in the highest amounts in the tonsillar carcinoma and it is confirmed that eNOS has an effect on programmed cell death (apoptosis) and cell proliferation (Li and Wogan, 2005; Fukumura et al., 2006; Lancaster and Xie, 2006). In our study we showed the levels of those two processes. To detect apoptosis we used cleaved caspase 3 as the marker. We found that apoptosis is not a very frequent event in the polypous or the tonsillar tissue. The highest levels were found in the germinative centers of the lymphatic follicles of the tonsils during chronic inflammation.

On the other hand, numerous proliferating cells were found among epithelial and stromal cells in the nasal polyp samples. The findings of Ki-67 positive endothelium in the inflamed mucosa suggested a stimulation of angiogenesis again. The positive results were constantly associated with the germinative centers of lymphatic follicles during tonsillar chronic inflammation.

The finding of fairly low amounts of the proliferating cells in the tonsillar carcinoma was highly interesting. Some Ki-67 positive cells were found in the germinative centers of the lymphatic follicles in the tonsillar carcinoma.

Our results confirmed the substantial role of eNOS on the pathological processes in the upper airway mucosa.

The levels of different subfamilies of HBD are also highly interesting. The results show that HBD-1 is involved in innate immunity and its levels do not react to inflammation or malignant transformation. HBD-2 was proven to be secreted in the inflamed tissue but in the tonsillar carcinoma samples it was not found in significant amounts. The production of HBD-3 in the tonsillar carcinoma is reduced in comparison to the levels in the inflamed tissue, but not so much as in the case of HBD-1 and 2.

These results show that cancerogenesis can be facilitated by a reduction in the production of HBD,

especially HBD-2, and also by producing large amounts of NO molecules by eNOS, which are involved in the processes of apoptosis, proliferation and cell cycle proliferation.

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