Original Article

Expression of Endothelial and Inducible Nitric Oxide Synthase and Caspase-3 in Tonsillar Cancer, Chronic Tonsillitis and Healthy Tonsils

(tonsillar cancer / chronic tonsillitis / eNOS / iNOS / caspase-3 / angiogenesis / apoptosis)

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Abstract. Neoangiogenesis and inhibition of apoptosis are two factors considered as major leading causes of tumorigenesis. NO, synthesized by NOS, plays an important role in tumour growth, dissemination and vascularization. Caspase-3 is an executive enzyme of apoptosis. The presented research work has been focused on the comparative evaluation of localization of the angiogenic and proapoptotic cytokines expressed in tonsillar diseases. The immunohistochemical reaction of eNOS, iNOS and caspase-3 in tonsillar cancer (N = 17), chronic tonsillitis (N = 11) and clinically healthy tonsils (N = 8) was detected. High eNOS occurrence in endothelial cells of highly vascularized regions in tonsillar cancer, variable eNOS expression in the vessels of lamina propria in chronic tonsillitis and high expression in the cytoplasm of endothelial cells of small veins in healthy tonsillar tissue was ascertained. Increased iNOS expression was found in cancer tissue in comparison with the healthy tonsils. Nevertheless, the highest expression of iNOS was found in chronic tonsillitis. Higher expression of caspase-3 was discovered in germinal centres of lymphoid follicles of the chronic tonsillitis tissue. However, the positivity in the interfollicular zone and surface squamous epithelium was weak only. Merely isolated caspase-3-positive cells were found in tonsillar cancer. Very low expression of caspase-3 was detected in the lymphatic follicles of the healthy tonsils. Research results showed high expression of eNOS in the carcinomatous tissue. The eNOS expression in chronic tonsillitis confirms its role in regulating the lymphocyte circulation. Low expression of caspase-3 in malignant epithelial cells of tonsillar cancer shows decreased capability of apoptosis compared to chronic tonsillitis tissue, where apoptosis seems to be rather frequent and concentrated in the germinal centres of lymphatic follicles. The differences in localization of eNOS and caspase-3 expression between benign and malignant processes may be a promising tool for precise morphological distinction of chronic inflammation and tumours.

Introduction

The tumour formation and mechanism of cell transformation are subjects of interest in molecular biology and oncology, with regard to the understanding of the oncogenetic process. Some physiological processes, including apoptosis, immunity reactions and angiogenesis, are considered to be important factors of tumour formation and development. Signalling and regulatory proteins of these processes have been specially studied.

Nitric oxide (NO) is a molecule playing an important role in the process of tumour growth, dissemination and vascularization (Gallo et al., 1998; Brennan et al., 2001). Hood et al. (1998) supposed NO to be an upstream signal for vascular endothelial growth factor (VEGF). The ability of tumour cells to induce new (blood and lymphatic) vessel growth is a critical factor in determining tumour size as well as regional and distant tumour spread (Folkman, 1995; Miyahara et al., 2007). NO is

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Abbreviations: eNOS – endothelial nitric oxide synthase, iNOS – inducible nitric oxide synthase, nNOS – neuronal nitric oxide synthase, NO – nitric oxide, NOS – nitric oxide synthase, OSAS – obstructive sleep apnoea syndrome, VEGF – vascular endothelial growth factor.

synthesized by three isoforms of nitric oxide synthase (NOS) – neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Brennan et al., 1999). A frequent occurrence of iNOS and eNOS was found in squamous cell cancers of head and neck (Gallo et al., 1998, 2002; Bentz et al., 1999).

Aberrant accumulations of cells, which could be the cause of malignant tumorigenesis, may be provoked by excessive proliferation of cells and insufficient apoptosis as well. In the healthy state, cell proliferation and cell death are in the right balance. Any deviation in cell death rate, proliferation intensity (or both) can be involved in disease pathogenesis (Saikumar et al., 1999). Cells that have developed irreparable genomic damage normally enter the apoptotic programme, part of the defence mechanism suppressing tumour growth. If this natural mechanism fails, an uncontrolled growth may occur and lead to initiation of tumorigenesis (Hickman, 2002). Caspase-3 was shown to be an executive enzyme of apoptosis. Both apoptotic molecular pathways (extrinsic and intrinsic) lead to the activation of caspase-3 (Tewari et al., 1995; Earnshaw et al., 1999; Segal and Beem, 2001). Therefore, the caspase-3 can be used for detection of apoptotic cells *in situ* and for assessment of the extent and impact of cell death in the respective tissue (Kucera et al., 2004; Jakob et al., 2008).

The presented study assignment was to find the expression of NO-releasing enzymes (eNOS and iNOS) and the expression of caspase-3 in the tissue of human tonsillar cancer, chronic tonsillitis and healthy tonsillar tissue. On the basis of results gained by executed research work it was possible to evaluate the level and localization of angiogenesis and apoptosis in the tissue. The comparative evaluation of cytokine expression in cancer disease with the expression in chronic tonsillitis and the comparison with healthy tonsillar tissue was the work's final aim.

Material and Methods

During the surgery, tissue specimens of palatine tonsils were obtained. Eleven specimens originated from patients suffering from chronic tonsillitis; 17 specimens were issued from patients affected with tonsillar squamous cell cancer. Specimens taken from eight patients with obstructive sleep apnoea syndrome (OSAS), proving clinically healthy tonsils and demonstrating no evidence of any tonsillar disease, were provided for comparative evaluation. Tissue specimens were fixed in 4% paraformaldehyde and embedded in paraffin. The production of eNOS, iNOS and caspase-3 was detected immunohistochemically. The three-step immunoperoxidase reaction was performed in tissue sections using anti-rabbit polyclonal NOS-3 (Santa Cruz Biotechnology, Santa Cruz, CA) 1 : 100, purified mouse anti-iNOS type II Mab (BD Transduction Lab., San Jose, CA) 1 : 100 and cleaved caspase-3 rabbit monoclonal antibody (Cell Signalling Technology, Danvers, MA) 1 : 200 as the primary antibody. As the secondary antibody, biotinylated goat-anti-rabbit IgG in PBS (Sigma-Aldrich, St. Louis, MO) 1 : 200 or goat-anti-mouse IgG in PBS (Sigma-Aldrich) 1 : 400 were used. Antibody binding was visualized by Vectastain ABC Elite kit peroxidase (VECTOR Lab., Burlingame, CA) and diaminobenzidine (DAB) peroxidase substrate solution (DAKO Cytomation, Glostrup, Denmark) as a chromogen. All tissue sections were counterstained with haematoxylin.

Results

The strongest eNOS positivity was found in capillary endothelial cells of highly vascularized regions of tonsillar cancer (Fig. 1). Variable expression of eNOS was detected in the vessels of tonsillar lamina propria in chronic tonsillitis. Strong eNOS immunoreaction was found in high endothelial venules (Fig. 2). In clinically

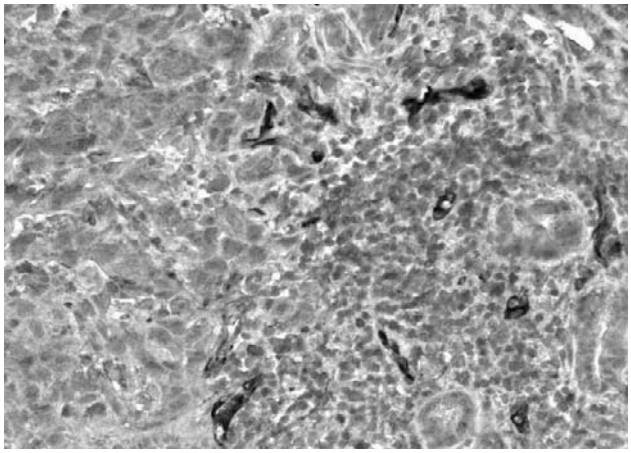

Fig. 1. Tonsillar cancer – detection of eNOS. The eNOS localization (brown colour reaction) in the endothelial lining of capillaries and small venules as well as in individual mononuclear cells in the connective tissue. Obj. magn. x40

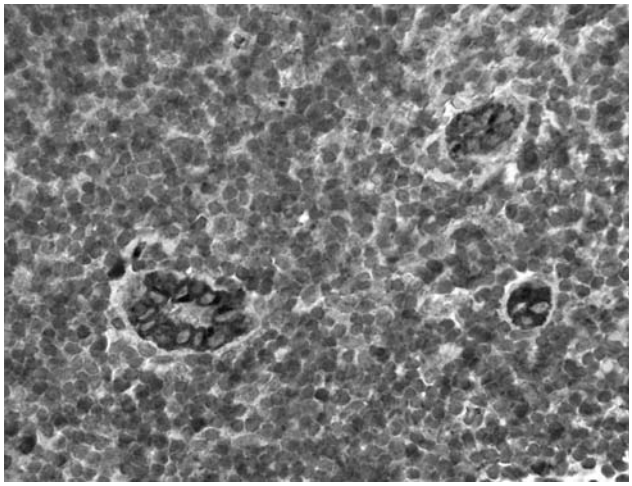

Fig. 2. Chronic tonsillitis – detection of eNOS. A high endothelial vein among lymphatic follicles. The reaction product (eNOS – brown granular accumulations) in the cytoplasm of tall endothelial cells. Obj. magn. x40

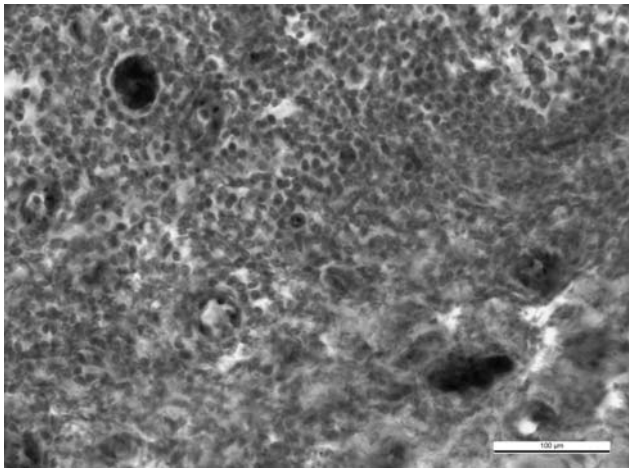

Fig. 3. Chronic tonsillitis – detection of iNOS, the positivity in the endothelium of small blood vessels in the lymphoid tissue. Obj. magn. x40

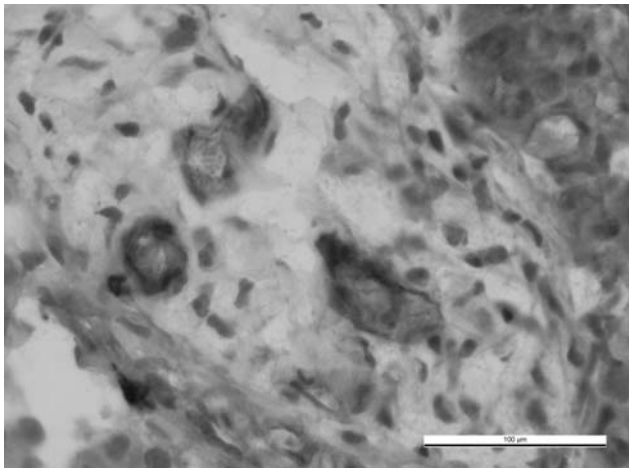

Fig. 5. Tonsillar cancer – detection of iNOS in some mononuclears of the connective tissue stroma of carcinomatous tonsil. Obj. magn. x63

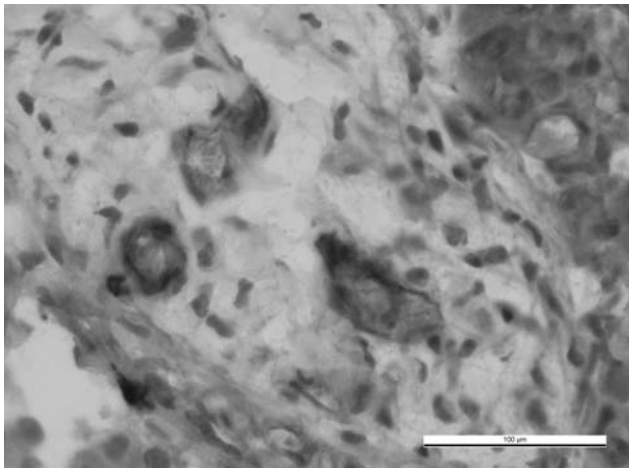

Fig. 4. Tonsillar cancer – detection of iNOS. The expression of iNOS in the endothelial cell cytoplasm of small stromal veins in tonsillar carcinoma. Obj. magn. x63

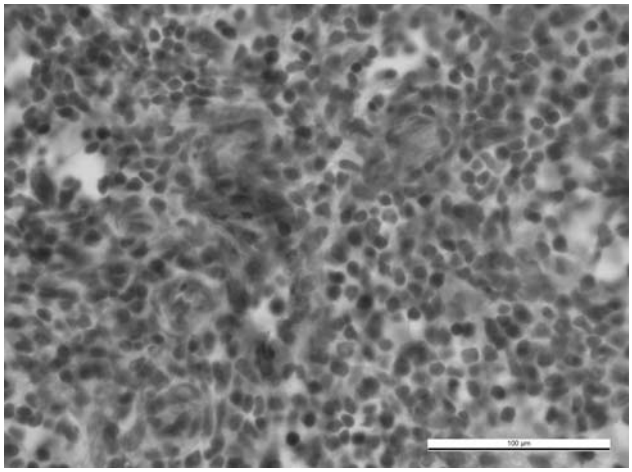

Fig. 6. Healthy tonsillar tissue – detection of iNOS. The reaction product demonstrating a positivity of iNOS in the endothelial cell cytoplasm of small veins in the lymphoid tissue. Obj. magn. x63

healthy tonsils, eNOS was detected in the cells of the superficial epithelium and at a high level in the cytoplasm of endothelial cells of small veins.

iNOS positivity was found in chronically inflamed, carcinomatous as well as clinically healthy tonsillar tissue. The strongest iNOS positivity, proved in the endothelial cytoplasm of small blood vessels in the lymphoid tissue (Fig. 3), was found in cases of chronic tonsillitis. In carciomatous tissue the reaction product of iNOS was found at a high level in the endothelial cell cytoplasm of small veins. A lower amount of the product was detected in the cytoplasm of some mononuclears in the connective tissue (Figs. 4, 5). The localization of iNOS reaction product in the clinically healthy tonsillar specimens was observed in the endothelial cell cytoplasm of small veins in the lymphatic follicles (Fig. 6).

Individual isolated caspase-3-positive cells were dispersed in tonsillar cancer (Fig. 7). In chronic tonsillitis, higher expression of caspase-3 in germinal centres of lymphoid follicles was detected. The positivity of this product in the interfollicular zone and surface squamous-cell epithelium of tonsils was rather weak (Fig. 8). Apoptotic processes in the clinically healthy tonsils are rare. In some specimens caspase-3 was located in the lymphatic follicles.

Discussion

Two important processes play a crucial role in the development of tumorigenesis:

a) increased cell proliferation followed by increased angiogenesis. The process is mediated by NO synthesized by NOS.
b) Inhibition of apoptosis – caspase-3 was shown to be an executive enzyme of apoptosis.

Reliable detection of cytokines and regulatory molecules involved in this processes could be a helpful tool in proper diagnosis of diseases, especially for distinguishing the chronic inflammation from cancer.

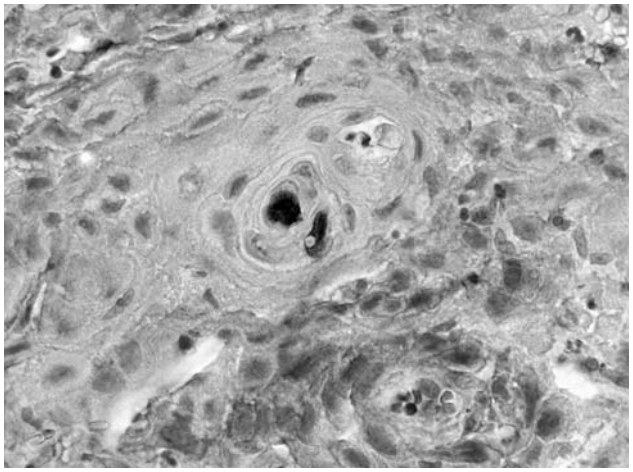

Fig. 7. Tonsillar cancer – detection of caspase-3. Forming keratin pearl in the tonsillar cancer. Expression of caspase-3 (brown signal) in several epithelial cells parabasally localized in the stratified squamous epithelium. Caspase-3 also in some mononuclear cells in the connective tissue of the lamina propria. Obj. magn. x63

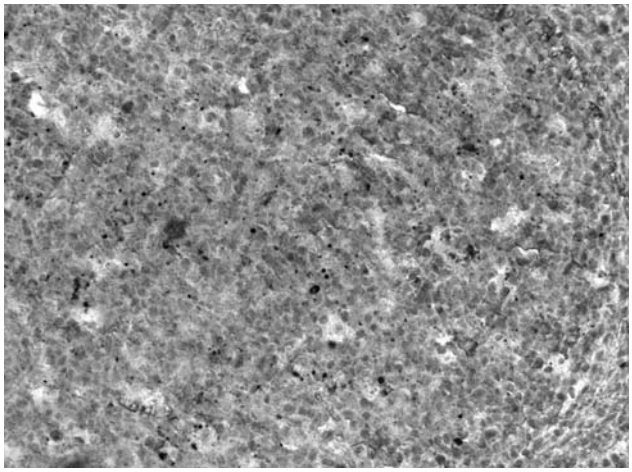

Fig. 8. Chronic tonsillitis – detection of caspase-3. Follicular aggregation of lymphocytes. Immunohistochemical detection of caspase-3 demonstrates localization in some mononuclear cells in the subepithelial connective tissue stroma. Obj. magn. x20

Since tumours cannot exceed 1–2 mm^3 of volume without developing new blood vessels, at an early point of development they must produce angiogenic factors (Folkman, 1990). Angiogenesis potentiated by NO was shown to play an important role in tumour progression and development of lymph node metastasis (Brennan et al., 2001). Nitric oxide is a signalling molecule produced by NOS in endothelial cells of vessels supplying the malignant tissue. The level of NOS expression indicates the intensity of angiogenesis (Gallo et al., 1998; Bentz et al., 1999; Brennan et al., 1999).

The special expression of two different NO synthases was compared morphologically. High intensity of angiogenesis in tonsillar squamous cell cancer with strong expression of both eNOS and iNOS in the endothelial cells was found and confirmed, similarly as in previously published data. The expression of eNOS in high endothelial venules of lamina propria in chronic tonsillitis also suggests the possibility of regulating the lymphocyte circulation. Different distribution of eNOS expression among different pathologies seems to be promising in distinguishing benign from malignant neoplasia.

Inhibition of apoptosis was shown to be one of the key factors helping tumour cells to evade the immune system response. Caspase-3 is a marker of apoptosis (Earnshaw et al., 1999). The process of apoptosis was found in isolated malignant epithelial cells of tonsillar cancer only. In chronically inflamed tonsils, apoptotic cells were detected in the group of maturing B lymphocytes in germinal centres of lymphoid follicles. Based on these findings, it may be presumed that cell proliferation accompanies the process of apoptosis under physiological conditions (maturing B lymphocytes). Malignant epithelial cells of tonsillar squamous cell cancer have a lower capability of apoptosis, suggesting the possibility of autonomous proliferation of malignant cells. Therefore, caspase-3 may be a suitable histological marker making it possible to distinguish chronic inflammation from a malignant process.

Table. Summary of morphological comparison of cytokine expression

Diagnosis	Expression of eNOS	Expression of iNOS	Localization of caspase-3
Tonsillar cancer	strongest expression in capillary endothelial cells of highly vascularized regions of tonsillar cancer	high expression in the endothelial cell cytoplasm of small veins low expression in the cytoplasm of some mononuclears in the connective tissue	isolated caspase-3-positive cells dispersed in carcinomatous tissue
Chronic tonsillitis	variable expression in vessels of tonsillar lamina propria strong expression in high endothelial venules	strong expression in the endothelial cytoplasm of small blood vessels in the lymphoid tissue	high expression in germinal centres of lymphoid follicles weak positivity in the interfollicular zone and surface squamous-cell epithelium
Healthy tonsils	weak expression in cells of the superficial epithelium high expression in the cytoplasm of endothelial cells of small veins	low expression in the endothelial cell cytoplasm of small veins in the lymphatic follicles	very low expression in the lymphatic follicles

The question of finding a suitable marker for diagnosis of malignant diseases still remains topical. According to the results of the presented research work it can be proposed that eNOS and caspase-3 immunohistochemical detection could be a useful tool for proper diagnosis of malignancies in tonsillar tissue.

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