

Distribution of 4-hydroxynonenal-protein conjugates as a marker of lipid peroxidation and parameter of malignancy in astrocytic and ependymal tumors of the brain

Gordana Juric-Sekhar¹, Kamelija Zarkovic¹, Georg Waeg², Ana Cipak³, and Neven Zarkovic³

¹Department of Neuropathology, Zagreb Clinical Hospital Center, Zagreb, Croatia; ²Karl Franzens University Graz, Institute of Molecular Biosciences, Graz, Austria; ³Institute "Rudjer Boskovic", Zagreb, Croatia

ABSTRACT

Aims and background. Lipid peroxidation (LPO) is an autocatalytic process caused by oxidative stress. It results in the production of 4-hydroxynonenal (HNE), which plays a crucial role in hypoxic brain injury, neuronal degeneration and apoptosis. The aim of this study was to evaluate the expression of HNE in 120 astrocytic and 40 ependymal tumors in relation to tumor type, grade of malignancy, angiogenesis, and presence of necrosis and apoptosis.

Methods. Immunohistochemical staining was performed using a monoclonal antibody for the detection of HNE-modified proteins.

Results. HNE-protein adducts were found in all tumors. The incidence of HNE-immunopositive tumor cells increased with increasing grades of malignancy. Significantly higher HNE expression was found in tumor cells of glioblastomas multiforme than in cells of pilocytic astrocytomas ($P < 0.005$), and in anaplastic ependymomas than in benign ependymomas ($P < 0.01$). HNE-immunopositive tumor cells were distributed more diffusely than in perivascular locations ($P < 0.05$). Pronounced HNE-protein adducts were detected in mitotic, necrotic, and apoptotic cells. HNE was expressed in the endothelium of almost all tumor vessels, but its expression in the walls of the vessels was significantly higher in diffuse and anaplastic astrocytomas than in pilocytic astrocytomas and glioblastomas multiforme ($P < 0.05$). The number of microvessels containing HNE in their endothelium and walls was significantly associated with the grade of malignancy in both astrocytic ($P < 0.001$) and ependymal tumors ($P < 0.05$), although microvessels in pilocytic astrocytomas were significantly more numerous ($P < 0.05$) than in diffuse astrocytomas.

Conclusions. LPO seems to be a common pathological process in astrocytic and ependymal glial tumors, proportional to the level of malignancy and neovascularization. Therefore, HNE might be involved in the damage of brain cells and the induction of malignancy.

Introduction

4-Hydroxynonenal (HNE) is a major product of lipid peroxidation (LPO), which is formed by radical-initiated degradation of ω -6-polyunsaturated fatty acids (PUFA) such as linoleic acid and arachidonic acid. The HNE level becomes elevated in cells under oxidative stress, and this aldehyde interacts with deoxyribonucleic acid (DNA) to form 4-hydroxynonenal deoxyguanosine (HNE-dG) adduct. Such HNE-dG adducts have also been found in various normal tissues of humans and rats¹⁻³. HNE forms relatively stable bioactive protein adducts and its biological effects are proportional to the intensity of its binding to the cellular proteins⁴. HNE is cytotoxic⁵, but it is also a

List of abbreviations

AA = anaplastic astrocytoma; AE = anaplastic ependymoma; CAT = catalase; CNS = central nervous system; DA = diffuse astrocytoma; DAB = diaminobenzidine; DNA = deoxyribonucleic acid; E = ependymoma; GBM = glioblastoma multiforme; GPx = glutathione peroxidase; GRx = glutathione reductase; GST = glutathione-S-transferase; HNE = 4-hydroxynonenal; HNE-dG = 4-hydroxynonenal deoxyguanosine; IMD = intratumoral microvessel density; LPO = lipid peroxidation; MDA = malondialdehyde; PA = pilocytic astrocytoma; PUFA = polyunsaturated fatty acid; ROS = reactive oxygen species; SOD = superoxide dismutase; VEGF = vascular endothelial growth factor

Key words: 4-hydroxynonenal, astrocytomas, ependymomas, angiogenesis.

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Correspondence to: Neven Žarkovic, MD, PhD, Institute "Rudjer Bošković", Bijenika 54, 10000 Zagreb, Croatia.
Tel +385-1-457 1234;
fax +385-1-456 1010;
e-mail zarkovic@irb.hr

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signaling molecule involved in the growth regulation interacting with cytokines and regulating expression of cellular oncogenes⁶⁻¹¹. Therefore, high reactivity of HNE with macromolecules and its multiple biological effects make this aldehyde a very attractive LPO product in the field of molecular oncology.

It is well known that the transition of normal cells to cancer is a complex, incompletely understood process that takes a long time. Permanent oxidative damage, overproduction of reactive oxygen species (ROS) and consequent macromolecular damage lead to initiation and promotion of cancer, while the vicious circle of carcinogenesis includes further local and oxidative stress that occurs in the case of cancer progression. Therefore, within its complex pathology cancer may be considered both a cause and a consequence of oxidative stress. Moreover, cocarcinogenic activities of substances that act as tumor promoters are associated with weakening of the cellular antioxidant defense system and a decrease in its constituents such as the endogenous key enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-S-transferase (GST), as well as antioxidant vitamins^{12,13}.

Cells in the central nervous system (CNS) have a relatively low level of antioxidant defense, a high iron content, extensive membrane turnover, a high lipid-to-protein ratio, and a high level of PUFA^{11,14,15}. Therefore, LPO is crucial for the development of CNS disorders and has been extensively studied in neurodegenerative diseases¹⁵⁻¹⁷. However, there have been only a few studies on LPO in CNS malignancies. Recent findings indicate local and systemic oxidative stress and LPO in patients with brain tumors, but some studies are contradictory^{9,18-21}. A pilot study of 47 different tumors in the brain showed a significant decrease in the erythrocyte glutathione reductase (GRx) and SOD activities in most types of brain tumors¹⁹. Another study revealed a higher activity of SOD and CAT in erythrocytes in patients with diffuse astrocytomas and glioblastomas as compared to the activity measured in healthy volunteers²⁰. Although these studies give contradictory results on the activities of the analyzed intracellular antioxidant enzymes in erythrocytes, they both show that CNS malignancies affect systemic antioxidant capacities. This is in agreement with the findings on systemic LPO in patients with astrocytomas^{18,21}. Serum and tissue concentrations of the LPO product malondialdehyde (MDA) were found to be increased in the malignant astrocytic tumor group compared to the low-grade astrocytic tumor group in one study¹⁸. 2-Hydroxyhexanal and HNE levels were higher in high-grade astrocytomas compared to low-grade astrocytomas and showed a negative correlation with survival²¹. These findings indicate that in CNS malignancies, like in many other types of malignant tumors, oxidative stress plays an important role in cancer development and progression both on a local and systemic level, while LPO analysis might be more

reliable than studies on intracellular antioxidants to evaluate the involvement of oxidative stress in cancer. Hence, improved knowledge of the mechanisms underlying the development of both benign and malignant tumors such as the implication of local and systemic oxidative stress may be very useful for cancer prevention, diagnosis and therapy.

The aim of this study was to evaluate the pathomorphological features of oxidative stress and LPO in different astrocytic and ependymal tumors by analyzing the presence of HNE-protein adducts and their correlation with the grade of malignancy.

Material and methods

Tumors

Surgical specimens from 160 glial tumors were analyzed at the Department of Neuropathology, Zagreb Clinical Hospital Center. The study included 120 astrocytic tumors [30 pilocytic astrocytomas (PA), WHO grade I; 30 diffuse astrocytomas (DA), WHO grade II; 30 anaplastic astrocytomas (AA), WHO grade III, and 30 glioblastomas multiforme (GBM), WHO grade IV] and 40 ependymal tumors [30 ependymomas (E), WHO grade II, and 10 anaplastic ependymomas (AE), WHO grade III]. All the tumors were classified according to the criteria of the WHO²².

Immunohistochemistry

Tumor tissues were fixed with formalin and embedded in paraffin. Immunohistochemical staining was performed using a monoclonal antibody for the detection of HNE-modified proteins. It was obtained from the culture medium of the clone derived from the fusion of Sp2-Ag8 myeloma cells with B cells of a BALBc mouse immunized by HNE-modified keyhole-limpet hemocyanin²³. The antibody is specific for the HNE-histidine epitope in HNE-protein (peptide) conjugates. For the immunohistochemical detection of the HNE-protein adducts the immunoperoxidase technique was used with secondary rabbit anti-mouse antibodies (DAKO, Glostrup, Denmark) applied on 4-5- μ m consecutive sections of the glial tumors. Finally, the sections were incubated with diaminobenzidine (DAB, Dako Glostrup, Denmark) substrate, and counterstained with hematoxylin (Kemika, Zagreb, Croatia) as described before⁸.

Estimation of HNE immunopositivity

Immunohistochemical investigation of HNE positivity was determined independently by 2 observers (GJS, KZ) and scored in a semiquantitative way (-0% positive cells, + <5% positive cells, ++ 5-25% positive cells, +++ 25-50% positive cells, ++++ >50% positive cells). The presence of HNE-protein adducts in specific structures

of the tumors (Rosenthal fibers, eosinophilic bodies), in tumor cells undergoing different vital processes (i.e., mitosis, apoptosis or necrosis) and in blood vessels was defined as negative (-) in the absence of HNE-protein adducts and as positive (+) in the presence of HNE-protein adducts.

Estimation of intratumoral microvessel density

Intratumoral microvessel density (IMD) was measured in microvessel "hot spots", i.e., microscopic areas containing the densest collections of microvessels first identified under low-power magnification. A vessel lumen was required for identification of a microvessel. Vessels with thin muscle walls were also included in the count. IMD was counted at $\times 400$ magnification using an eyepiece graticule (the graticule covered an area of 0.22 mm^2). Five adjacent fields were counted within each hot spot²⁴. IMD was measured according to 3 parameters: 1) total number of microvessels counted per square millimeter; 2) number of microvessels with HNE-protein adducts only in their walls (outside the endothelial layer) per square millimeter; 3) number of microvessels with HNE-protein adducts only in their endothelium per square millimeter.

Statistics

The incidence of HNE-positive *versus* HNE-negative tissues depending on the type of tumor was evaluated using the chi-square test. Possible differences in staining intensity were determined with the Mann-Whitney test, using numerical description of positivity corresponding to the respective standard grading of positivity as described above. Differences were considered statistically significant at $P < 0.05$. The SPSS 11.0 statistical program for Windows was used to analyze the results obtained.

Results

The presence of HNE-protein adducts was detected in all tumors and the incidence of HNE-immunopositive tumor cells increased with the grade of tumor malignancy (Figure 1). A significantly greater presence of HNE-protein adducts was found in tumor cells of GBM than PA ($P < 0.005$) as well as in tumor cells of AE than E ($P < 0.01$). However, there was no difference between GBM, AA and DA (for all, $P > 0.1$). HNE-protein adducts were expressed in a vast majority of the tumor cells of AE compared to all other tumors ($P < 0.01$ for all tumors). Benign ependymomas of grade I (E) had almost the same intensity of HNE-protein adducts as GBM ($P > 0.1$).

Perivascular distribution of HNE-immunopositive tumor cells (Table 1) was seen mostly in PA and E (Figure 2A and 2E), while a significantly higher incidence of dif-

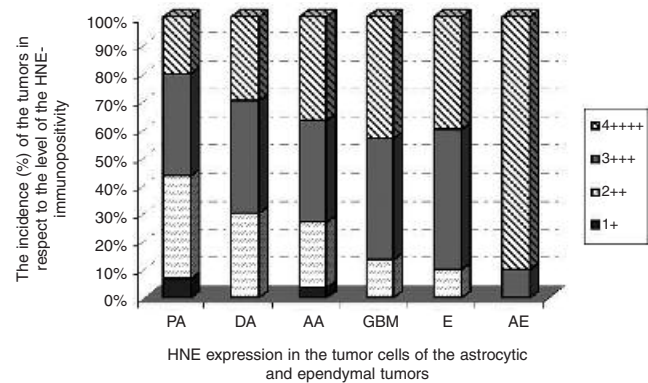


Figure 1 - Incidence of HNE-protein adducts in tumor cells of astrocytic and ependymal tumors. Number of tumors analyzed: all astrocytic tumors as well as ependymomas ($n = 30$) and anaplastic ependymomas ($n = 10$).

Table 1 - Distribution of HNE-protein adducts in astrocytic and ependymal tumor cells

Tumor	Distribution of HNE-immunopositive tumor cells		Total number of tumors
	Perivascular*	Diffuse**	
PA	15 (50%)	15 (50%)	30
DA	1 (3%)	29*** (97%)	30
AA	5 (17%)	25*** (83%)	30
GBM	5 (17%)	25*** (83%)	30
E	11 (37%)	19 (63%)	30
AE	0	10*** (100%)	10

PA, pilocytic astrocytoma; DA, diffuse astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; E, ependymoma; AE, anaplastic ependymoma.

*HNE-immunopositive tumor cells found only around blood vessels; **HNE-immunopositive tumor cells found throughout tumor tissue not exclusively around blood vessels; ***Significantly higher incidence of diffuse immunopositivity (chi-square).

fuse immunopositivity ($P < 0.05$) was found in the other tumors, including malignant ependymomas, AE.

A marked presence of HNE-protein adducts was noticed in eosinophilic bodies in a vast majority of PA (80%) (Figure 2B, Table 2). Moreover, necrotic areas in GBM (Figure 2D) and in AE expressed HNE. Sporadic apoptosis was found in 21 of 30 GBM as well as in 1 AE, and all of them showed the presence of intracellular HNE-protein adducts. Mitotic figures seen in AA, GBM, and AE were also positively HNE immunostained (Figure 2F). Endothelium of the blood vessels expressed HNE in almost all tumors irrespective of their type and grade, whereas the presence of HNE-protein adducts in vessel walls, outside the endothelial layer, varied depending on the type of tumor (Figure 2A-E). A significantly higher incidence of HNE immunopositivity in the

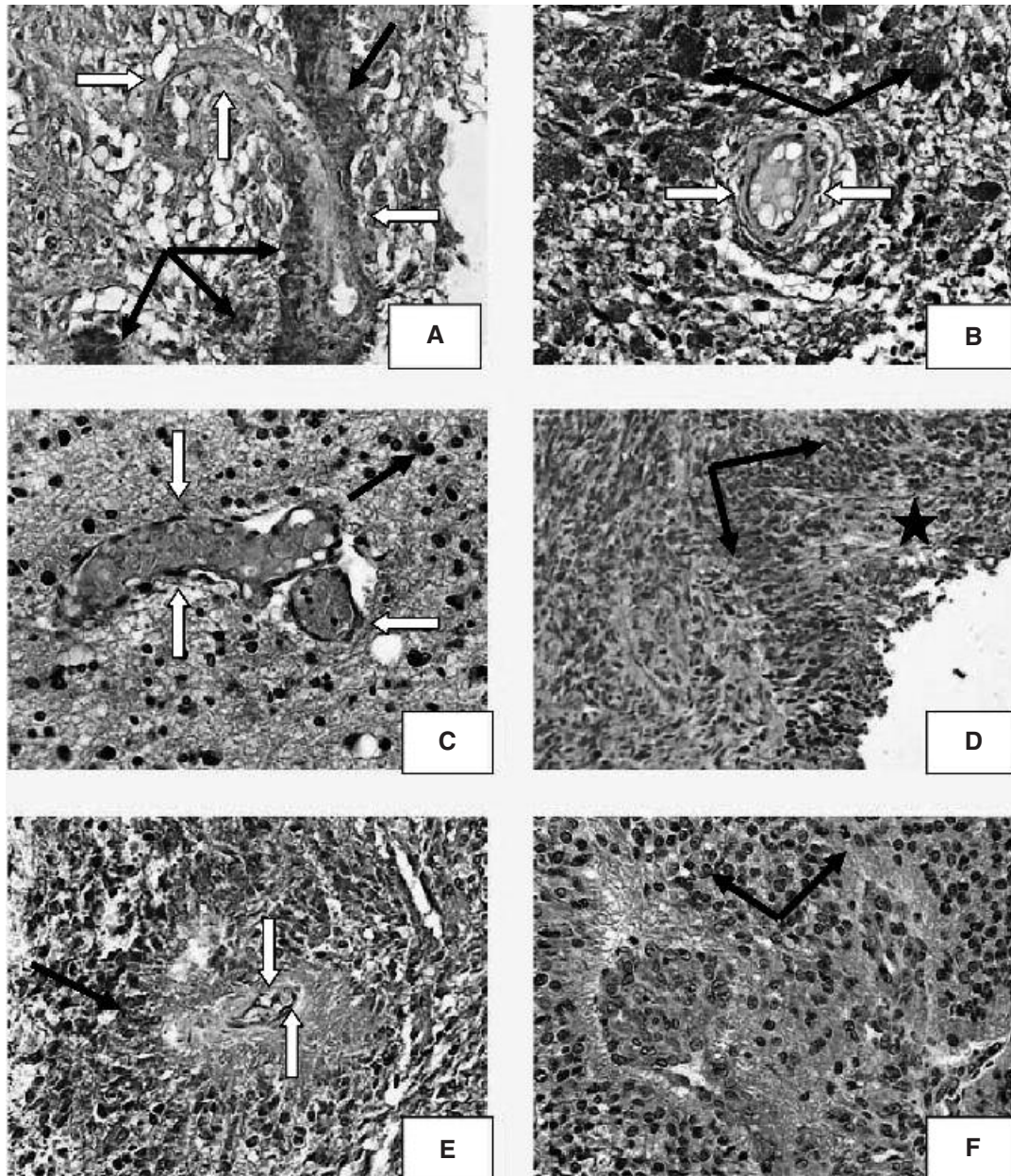


Figure 2 - Immunohistochemical findings of HNE-protein adducts. A) Pilocytic astrocytoma with perivascular distribution of HNE-immunopositive tumor cells (black arrow), as well as B) strong immunoreactivity for HNE in eosinophilic bodies. C) Diffuse astrocytoma with single tumor cells showing expression of HNE (black arrow). D) Glioblastoma multiforme: numerous tumor cells and necrotic area (black star) showing immunoreactivity for HNE. E) Strong expression of HNE in tumor cells of a rosette in ependymoma. F) Strong expression of HNE in tumor cells and mitosis (black arrow) in anaplastic ependymoma. In 2A the endothelium of the blood vessel is negative, while its wall is immunopositive for HNE (white arrow); in photos 2B and 2C the endothelium of the blood vessels is HNE-immunopositive, while the wall is negative (white arrow). Magnification: A, B, C, E, F, $\times 400$; D, $\times 200$.

remainder of the vessel wall was found in DA and AA compared to PA and GBM ($P < 0.05$).

IMD values gradually increased from DA to GBM, and from E to AE (Table 3). Significantly higher IMD values were seen in AA versus DA ($P < 0.05$, except for the value of the microvessels with HNE-immunopositive walls), and in GBM versus DA, and GBM versus AA ($P < 0.001$).

IMD was significantly higher in AE than in AA ($P < 0.001$). Moreover, IMD values were significantly higher in PA versus DA ($P < 0.05$), and in PA versus AA ($P < 0.05$ only for the values of the total number of all microvessels observed), while IMD values in GBM were significantly higher than in PA ($P < 0.05$, except for HNE-immunopositive walls of the microvessels).

Table 2 - Presence of HNE-protein adducts in specific tissues and cells of astrocytic and ependymal tumors

Parameter	HNE immunopositivity in tumor					
	PA	DA	AA	GBM	E	AE
Rosenthal fibers						
Positive*	-					
Negative**	30 (100%)					
Eosinophilic body						
Positive	24 (80%)					
Negative	-					
Not found***	6 (20%)					
Endothelium of the vessels						
Positive	29 (97%)	30 (100%)	26 (97%)	28 (93%)	30 (100%)	9 (90%)
Negative	1 (3%)		4 (13%)	2 (7%)		1 (10%)
Wall of the vessels						
Positive	17 (57%)	24 (97%)	22 (73%)	17 (57%)	19 (63%)	7 (70%)
Negative	13 (43%)	6 (13%)	8 (27%)	13 (43%)	11 (37%)	3 (30%)
Mitotic tumor cells						
Positive	-	-	18 (60%)	25 (83%)	-	6 (60%)
Negative	-	-	-	-	-	-
Not found	30 (100%)	30 (100%)	12 (40%)	5 (17%)	30 (100%)	4 (40%)
Tumor necrosis						
Positive	-	-	-	30 (100%)	-	9 (90%)
Negative	-	-	-	-	-	-
Not found	30 (100%)	30 (100%)	30 (100%)	-	30 (100%)	1 (10%)
Apoptotic cells						
Positive	-	-	-	21 (70%)	-	1 (10%)
Negative	-	-	-	-	-	-
Not found	30 (100%)	30 (100%)	30 (100%)	9 (30%)	30 (100%)	9 (90%)

PA, pilocytic astrocytoma; DA, diffuse astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; E, ependymoma; AE, anaplastic ependymoma; HNE, 4-hydroxynoneal.

*Positive, pronounced HNE immunopositivity; **Negative, HNE immunopositivity was not observed; ***Not found – particular cells or structures were not found.

Discussion

Oxidative stress occurs due to the production of ROS in tissues and the inability of a biological system to neutralize and eliminate them. The excess ROS can damage cellular lipids, protein or DNA^{12,13}. LPO is a process where free radicals attack PUFAs in cell membranes, resulting in cell damage^{2,12,13}. Oxidative stress and LPO have been suspected to be involved in many diseases^{5,15-17,25-28}, as well as in carcinogenesis^{13,29-31}. However, the role of oxidative stress in malignancies may not be limited to early mutagenic events^{13,30,31}. It also promotes cell proliferation by activating growth-promoting signaling pathways³¹. The underlying mechanisms remain unclear. Human tumor cell lines *in vitro* produce ROS at a far greater rate than do non-transformed cell lines, thereby exerting persistent oxidative stress²⁵. On the other hand, the mechanisms of the resistance of cancer cells to oxidative stress are still unknown. In the brains of immobilized rats oxidative stress was generated by decreasing the activities of SOD, GST and CAT, while increasing LPO³². Similar mechanisms could be present in human brain tumors. Hence, cancer may be considered as a disease of complex pathology where ox-

idative stress could play an important role in tumor development and progression, being induced by carcinogenic oxygen free radicals generating itself as a result of persistent oxidative stress^{13,29}. The LPO product HNE,

Table 3 - Intratumoral microvessel density in the astrocytic and ependymal tumors

Tumor	IMD/mm ² (average ± SD)*		
	Total number	HNE-Immunopositivity	
		Wall	Endothelium
PA	24.5 ± 4.1	12.4 ± 7.3	18.7 ± 5.9
DA	15.5 ± 3.7	7.4 ± 3.5	11.6 ± 4.1
AA	20.1 ± 5.1	10.5 ± 5.1	15.5 ± 4.3
GBM	27.9 ± 4.4	16.8 ± 5.7	25.7 ± 5.9
E	13.9 ± 3.1	5.9 ± 2.7	12.1 ± 3.6
AE	29.5 ± 2.3	22.6 ± 4.4	28.9 ± 2.4

IMD, intratumoral microvessel density; PA, pilocytic astrocytoma; DA, diffuse astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; E, ependymoma; AE, anaplastic ependymoma
* Five adjacent fields were counted within each "hot spot" of the section for 10 (AE) or 30 tumors per group (all other types of tumors).

which is well known as a “second messenger of free radicals” and is considered to be one of the major bioactive markers of LPO¹¹, regulates a wide variety of cellular processes and may be an important factor involved in tumor progression^{11,26}.

In our study, HNE immunopositivity was found in all astrocytic and ependymal tumors of the brain. Our findings of abundant HNE in astrocytic and ependymal glial tumor tissues could reflect severe and long-lasting oxidative stress, which allowed generation of diffusely scattered HNE-protein adducts. HNE causes impairment of the blood-brain barrier^{8,14,25,33} and may cause further spread of LPO from blood into the brain and vice versa^{25,26}, as well as a more pronounced local distribution of LPO generated in one area.

In our study, the incidence of HNE-immunopositive tumor cells increased with the grade of tumor malignancy. However, an exception to this general rule was the much greater HNE expression in cells of all ependymal tumors. Benign ependymomas had similar HNE expression as GBM, and AE showed even greater expression than GBM. It is possible that the ependymal origin makes these tumor cells more prone to LPO.

Detection of HNE expression in mitotic figures in high-grade astrocytic and ependymal tumors could reflect effects of HNE on cell growth. HNE interferes with the activity of cytokines and acts in cell signaling^{11,34}, while its binding to the cellular proteins is essential for its effects on cell growth⁴.

The presence of HNE even in small areas of necrosis found in GBM and AE indicates that the onset of necrosis could be caused by high amounts of HNE^{4,35-38}. Various agents (oxidants, xenobiotics, radiation, etc.) induce LPO, leading to the formation of HNE³⁹. Cytotoxicity of HNE could be involved in the initiation of apoptosis because reduced concentrations of GSH are found in apoptotic cells⁴⁰. It could be one of the reasons for the HNE immunopositivity detected in apoptotic bodies of high-grade astrocytic and ependymal tumors.

Blood vessels may play an important role in the development of oxidative stress and consequently LPO in brain tumors. In our study, HNE was expressed in the endothelium of almost all tumor vessels. However, HNE immunopositivity was seen in the vessel walls of some astrocytic and ependymal tumors. It was reported that HNE induces vascular endothelial growth factor (VEGF) in retinal pigment epithelial cells. The mechanism of this important activity of HNE involves depletion of intracellular GST and an increase in oxidative stress, leading to increased VEGF mRNA⁴¹. We assume that a similar mechanism may play a role in glial tumors. In our study the number of microvessels containing HNE in the endothelium and the remainder of the wall increased with the grade of malignancy. The persistent LPO mediated through HNE may be a mechanism causing the growth of tumors. Malignant brain tumors are characterized by extensive neovascularization; this

would be attributed to the high levels of VEGF, which may have been partially induced by HNE. This possibility is further supported by a study carried out by Hovington *et al.*⁴², who described that irradiation, which causes oxidative stress and LPO, enhances VEGF secretion in tested glioblastoma cell lines and increases the angiogenic potential of the tumor. On the other hand, angiogenic factors other than VEGF may also contribute to vascular proliferation in low-grade astrocytomas⁴³. High values of microvessel density observed in PA with pronounced HNE immunopositivity would suggest that HNE may be considered among the factors responsible for angiogenesis in low-grade gliomas as well.

To summarize, our findings suggest that oxidative stress, and HNE in particular, is involved in the development of CNS tumors, in particular malignant tumors. Further study is necessary to investigate the mechanisms of HNE-induced damage in cells and the induction of glial tumors of the brain.

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