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Expression of Inducible Nitric Oxide Synthase (iNOS) in Astrocytomas of Various WHO Grades with and without Malignant Progression

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Abstract

Nitric Oxide (NO), a free radical gas implicated in a wide variety of biological processes, is generated in many mammalian cells by a family of enzymes, i.e. Nitric Oxide Synthases (NOS). Nitric oxide produced by the inducible NOS isoform (iNOS or NOS II) seems to play an important role in tumor biology showing both tumor promoter and antitumor activity.

The aim of the present study was to determine the cellular localization of iNOS in astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III) and glioblastoma (GBM) (WHO grade IV) by immunohistochemistry using commercially available antibodies, as well as to detect possible changes in astrocytomas with malignant progression compared to the non-progressive group.

Positive iNOS immunostaining was detected in all samples of astrocytoma specimens, whereas non-tumorous brain tissue adjacent to the tumor did not show any iNOS positivity. The tumor tissue revealed a highly inhomogeneous staining pattern. We found uniformly stained tumor specimens and groups of markedly iNOS-positive tumor cells, besides unstained tumor tissue as well as randomly scattered individual tumor cells expressing a marked staining. Immunoreactivity was located within the cytoplasm of neoplastic astrocytes.There were no statistically significant differences among astrocytomas of WHO grades II, III, and IV, between astrocytomas with and without malignant progression. Furthermore, there was no clear correlation between the density of iNOS-immunopositive cells and survival time.

Further studies will be necessary to elucidate the role of iNOS in concert with other signaling pathways played in astrocytomas in order to see how and to what extent they are functionally interrelated.

Keywords: Inducible nitric oxide synthase (iNOS); Astrocytomas; Glioblastoma; Malignant progression

Introduction

Nitric Oxide (NO) is a short-lived pleiotropic biomolecule with a multitude of biologic functions. Since its discovery as a biologically active molecule in the late 1980s, NO is thought to play a role as a signal molecule in many organs, in immunological and defense mechanisms [1-3], in multiple signal transduction pathways [1,2], as well as in carcinogenesis [2,4-9]. Under physiological conditions, NO can travel long distances in order to act as an intercellular messenger in the brain. Its targets include adjacent neurons and astrocytes. Opposite effects of NO, harmful as well as protective, have been observed [10]. NO is a product of the conversion of L-arginine to L-citrulline by Nitric Oxide Synthase (NOS), which exists as three enzyme classes: the calcium-dependent endothelial isoform (eNOS or NOS III), the neuronal or brain isoform (nNOS or NOS I), and a calcium-independent inducible or immunologic isoform (iNOS or NOS II) [11]. At the present time, the role of NO during tumor development reveals a complex picture. It may be found in macrophages and microglial cells, in hepatocytes, neutrophils, endothelial cells, and astrocytes. This form is important in the tumoricidal activity of T lymphocytes and the bacteriostatic response of reticuloendothelial cells [3].

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The observation that levels of calcium-independent NOS (iNOS) are higher in malignant tissue, and the localization of iNOS to intratumoral macrophages and microglial cells or endothelial cells of the tumor vasculature suggests that the intratumoral environment of these cancers results in the induction of this special isoform (iNOS) [12,13,15-18]. Studies showing an increase in the growth rate, vascular density and invasiveness of a human tumor cell line transfected to constitutively express iNOS support this assumption [19-21]. Consistently, administration of a highly selective inhibitor of iNOS limited the invasion and growth rate of iNOS transfected tumor cell lines as well as of other tumors expressing this isoform [22]. In addition it was shown that lipoproteins can induce the formation of reactive astrocytes, inducing iNOS giving experimental support to a role played by LDL and HDL inducing a reactive response [8]. Moreover, NO was reported to play a critical role in neurodegenerative disorders [23,24]. So far, iNOS has been detected in tumor cells of the human brain, breast, lung, stomach, colon, prostate, cervix, ovary, kidney, liver, pancreas, and urinary bladder, among others [13,15-17,25-36].

Astroglial tumors are the most frequently encountered brain tumors. They are tumors of neuroepithelial tissue. For each tumor entity, predicting the biological behaviour by means of histological grading was introduced by the WHO. Astrocytoma (WHO grade II) is a diffusely infiltrating tumor that typically affects young adults and is characterized by a high degree of cellular differentiation and slow growth; the tumor occurs throughout the CNS but is preferentially located supratentorially and has an intrinsic tendency for malignant progression to anaplastic astrocytoma and, ultimately, glioblastoma. Anaplastic astrocytoma (WHO grade III) is a diffusely infiltrating, malignant astrocytoma that primarily affects adults, preferentially located in the cerebral hemispheres, and which is histologically characterized by nuclear atypia, increased cellularity and significant proliferative activity. The tumor may arise from diffuse astrocytoma WHO grade II or *de novo*, i.e. without evidence of a less malignant precursor lesion, and has an inherent tendency to undergo progression. Glioblastoma or Glioblastoma Multiforme (GBM) (WHO grade IV) is a highly malignant neuroectodermal tumor composed of densely packed, anaplastic, and highly dedifferentiated tumor cells making the histogenetic typing difficult.

Several pathophysiological properties important for tumor cell survival and tumor pathology may be mediated by NO. Recent studies have suggested a role of NO in causing increased tumor blood flow, edema, and vascular permeability [4-6,37]. These features of tumors are particularly prominent in pathologically high-grade tumors of the CNS. Furthermore, cytokines found in brain tumors, such as interleukin 1, tumor necrosis factor alpha, and gamma-interferon induce NOS activity *in vitro* [7,38]. Up to now, little is known about the expression of iNOS in astrocytomas or in progressive astrocytomas. An increased expression of the brain and endothelial forms of NOS (NOS I and NOS III, respectively) in astrocytic tumors was described by [31]; the highest levels of expression were found in higher grade tumors. Each of these two isoforms was found in tumor cells and tumor endothelial cells. The macrophage isoform of NOS was less frequently detected and expressed at a lower level, predominantly in tumor endothelial cells. NADPH diaphorase staining for NOS activity paralleled this pattern of NOS expression.

In the present study, the local evidence of iNOS in tumor tissue of astrocytomas without and with malignant progression was investigated immunohistochemically using a commercially available iNOS antibody and by Western blotting technique. The study was aimed to compare iNOS expression in astrocytomas of different grades and behavior in order to discuss its potential roles in brain tumor biology.

Table 1: Characteristics of the investigated astrocytomas with malignant progression.

Pat	Age	Gender	Grade	Interval in years between surgical interventions
1	26	Female	2→3	3
2	48	Female	2→4	3
3	26	Male	2→4	3
4	77	Male	2→4	1.5
5	57	Male	2→4	1.5
6	49	Female	2→2→4	4 and 0.3
7	45	Male	2→3	2.5
8	31	Male	2→2→3	2 and 2
9	32	Female	2→3	4
10	21	Male	3→4	2
11	41	Male	2→4	4
12	30	Female	2→2→3	1 and 3
13	32	Male	2→4	1.5

Materials and Methods

Tissues from the following brain tumor entities were investigated: (a) 11 cases of astrocytomas WHO grade II, (b) 10 cases of anaplastic astrocytomas WHO grade III, (c) 17 cases of Glioblastoma Multiforme (GBM) WHO grade IV, and (d) 12 cases of astrocytomas showing malignant progression. Detailed information about the patients with tumor progression is provided in Table 1. All patients received the appropriate, state-of the art treatment.

Immunohistochemistry

Resected specimens were fixed in 4% formaldehyde and embedded in paraffin. Deparaffinized and rehydrated 4µm thick sections were washed in tap water, then in distilled water and finally in 0.05M Tris buffer (pH 7.6). Blocking of unspecific binding sites was done with a commercially available protein blocking agent (Ultra-Tech., Coulter-Immunotech, Marseille, France) for 10 min. After removing the excess blocking reagent, sections were incubated with the primary polyclonal rabbit antibody (Transduction Laboratories, Biomol, Hamburg, Germany), diluted to 1:1200 in RPMI-medium (Life Technologies, Eckenstein, Germany). The antibody is directed against the mouse iNOS C-terminal peptide (1131-1144) plus additional N-terminal Cys conjugated to KLH (CKKGSALEEPKATRL). According to the manufacturer, the antibody recognizes the iNOS 130 kDa protein in humans, rats and mice without cross-reaction to eNOS or nNOS. For negative control, the sections were incubated with RPMI-medium alone. Colorectal cancer specimens, likewise formalin-fixed and paraffin embedded, were used as positive control for the iNOS antigen in immunohistochemistry (Wolff "et al". 2000).

After an 18h incubation of the primary antibody at 4°C, sections were rinsed with Tris buffer and incubated with a secondary, biotinylated goat anti-rabbit antibody (Vectastain, ABC-AP Elite Kit; Vector Laboratories, Burlingame, USA). After incubation with Vectastain ABC-AP reagent (avidin-biotin-complex-alkaline phosphatase, as described by the manufacturer), sections were stained

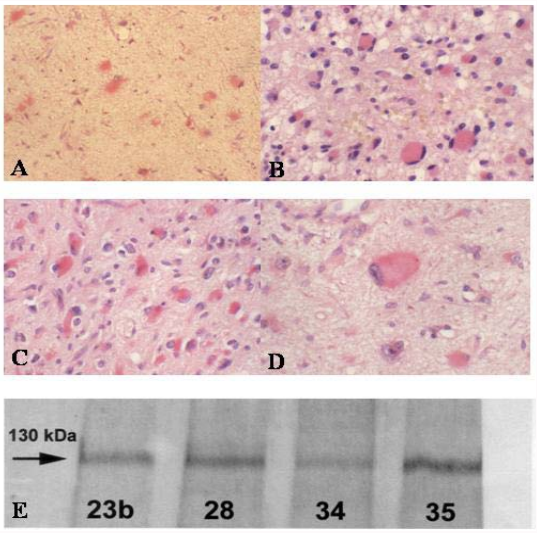

Figure 1: Immunoreactivities for iNOS in specimens of astrocytoma WHO grade II (A), anaplastic astrocytoma WHO Grade III (B), and glioblastoma multiforme WHO grade IV (C, D) (avidin-biotin complex, alkaline phosphatase, ABC-AP, counterstained with hematoxylin). (E) Band at 130kDa showing the presence of iNOS in tissues of four cases of GBM WHO grade IV on Western blot analysis.

with the Fast Red chromogen system (Coulter-Immunotech.) for 15 min, counterstained with hematoxylin and mounted with glycerol.

In frozen specimens of six GBMs, the NOS activity was co-localized by the NADPH-diaphorase reaction according to [39].

Western blotting

Protein homogenates from brain tumor specimens were prepared in 10 volumes of buffer A (25mm Tris-HCl, pH 7.4-100mm NaCl EDTA-1nm [ethylenebis(oxyethyl-enenitrilo)] tetraacetic acid-1mm phenylmethylsulfonyl fluoride). Following centrifugation at 15,000 rpm for 20 min, soluble extracts were partially purified using 2',5'-ADP agarose chromatography as described [40]. Affinity-purified samples were fractionated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with the iNOS antibody used for immunohistochemistry (dilution 1:1200). Immunoreactive species were visualized by enhanced chemoluminescence.

Morphometry

The morphometrical evaluation was made at a 400x magnification with careful registration of the morphological features. The numerical density of immunopositive cells, of immunonegative cells, and of all cells was determined following the 'random systematic sampling' [41]. Briefly, in 'random systematic sampling', the first measuring field was positioned randomly within the structure of interest: the next measuring field was then positioned systematically adjacent to the previous one; every second measuring field was considered resulting in a total of ten measuring fields. Further details of the morphometric methods have been described previously [41]. While counting the cells, the rules of the unbiased grid were applied. Briefly, two lines of the measuring field were defined as forbidden lines. All cell profiles hitting the forbidden lines as well as their extensions were not counted. All cells falling within the measuring field or touching the non-forbidden lines and their extensions were counted [42]. The numerical density was calculated as the number of cells per square millimeter (n/mm^2), and the Labeling Index (LI) expressed in percent, i.e. the number of immunopositive cells divided by the number of all cells multiplied by 100, was also determined. The numerical density of "all cells", i.e. the number of immunonegative cells plus the number of immunopositive cells, was also calculated.

Statistics

The evaluated data were processed using the Statistical Package for the Social Sciences (SPSS). Correlation, one-way analysis of variance (ANOVA), Chi-square statistics, Student's t-test, and the non-parametric Mann-Whitney U-test were used.

Results

The clinicopathological data (i.e. age of the patient, gender WHO grade, change of grade with progression, interval in years between surgical interventions) of the examined astrocytoma cases with malignant progression are given in Table 1.

Positive iNOS immunostaining was detected in all samples of tumor tissue (Figure 1). The presence of iNOS in the tumor tissue was also detected by Western blotting technique showing a band of 130 kDa (Figure 1E). In the negative control, immunoreactivity was completely lacking, while the positive control (colorectal carcinoma) displayed a clear-cut immunopositivity, which was co-localized with NADPH-diaphorase activity of the tumor cells.

Immunoreactive tumor cells were both, diffusely or focally, distributed throughout the tumor tissue. The immunoreaction was seen homogeneously only in the cytoplasm of the astrocytic tumor cells. In general, it has to be noted that for all examined groups the quantitative data showed a high interindividual variability reflected by high values of the standard deviation and the standard error of the mean.

Astrocytomas without progression

When analyzing the overall differences among astrocytomas of grades II, III, and IV, no significant differences were seen on ANOVA analysis for the numerical density of all cells, immunopositive or immunonegative cells, as well as for the labeling index (Table 2). A post-hoc analysis comparing the three grades separately with each other did also not show significant differences between grades II and III, grades III and IV, and grades II and IV although the numerical density of immunopositive cells was increased in the groups with the higher grades.

Table 2: Numerical density (n/mm^2) of immunopositive (im-pos) cells and immunonegative (im-neg) cells and labeling index (%) in astrocytomas without Malignant Progression.

	Grade II		Grade III		Grade IV	
	Mean	Sem	Mean	Sem	Mean	Sem
all cells	2784.00	396.49	4232.22	365.09	3529.33	487.31
im-pos cells	214.00	84.77	405.77	175.80	401.33	159.00
im-neg cells	2570.00	365.98	3624.44	522.52	3128.00	515.60
LI	6.57	2.71	17.05	6.94	12.70	4.80
p-values	ANOVA	II/III	III/IV	II/IV		
all cells	.14	.02	.26	.24		
im-pos cells	.62	.34	.98	.31		
im-neg cells	.40	.12	.50	.38		
LI	.41	.18	.61	.27		

Table 3: Numerical density (n/mm²) of immunopositive (im-pos) cells and immunonegative (im-neg) cells and labeling index (%) in astrocytomas with malignant progression: differences among grades II, III, and IV.

	Grade II		Grade III		Grade IV	
	Mean	Sem	Mean	Sem	Mean	Sem
all cells	2298.18	429.42	2923.33	504.95	3723.75	581.04
im-pos cells	337.27	196.50	521.66	222.95	976.25	315.50
im-neg cells	1960.90	381.22	2401.66	507.84	2747.50	406.02
LI	12.00	5.41	18.38	9.62	23.87	6.65
p-values	ANOVA	II/III	III/IV	II/IV		
all cells	.13	.36	.32	.07		
im-pos cells	.17	.54	.26	.11		
im-neg cells	.39	.50	.61	.17		
LI	.44	.57	.65	.18		

Astrocytomas with progression

When analyzing the overall differences between grades II, III, and IV in astrocytomas with malignant progression, no significant differences were seen by ANOVA analysis for the numerical density of all cells, immunopositive or immunonegative cells, as well as for the labeling index (Table 3). A post-hoc analysis comparing the three grades did also not show significant differences between grades II and III, grades III and IV, and grades II and IV although the numerical density of immunopositive cells was increased in the groups with the higher grades.

Differences between progression versus no progression

The comparison of the tumor group with progression with that without progression for all grades (i.e. II, III, IV) did show a significant increase in the numerical density of immunopositive cells (Table 4). When assessing the differences between these two groups, by analyzing the three WHO grades separately, no significant differences between the progressive and the non-progressive group were noted (Table 4).

Number of operations

Within the group of astrocytomas with malignant progression, no significant differences were noted between the different number of operations (Table 5). When comparing the first operation with the subsequent operations grouped together in one group, no significant differences were obvious.

Correlation with survival

No significant correlation could be drawn between the evaluated morphometric parameters and survival time.

Table 4: Numerical density (n/mm²) of immunopositive (im-pos) cells and immunonegative (im-neg) cells and labeling index (%) in astrocytomas: progression versus no progression.

	Progression		No Progression		
All Grades	Mean	Sem	Mean	Sem	P-value
all cells	2904.40	306.20	3496.17	272.93	.15
im-pos cells	586.00	148.53	347.41	86.70	.17
im-neg cells	2318.40	244.52	3095.29	287.64	.04
LI	17.33	3.90	12.05	2.91	.28
Grade II					
all cells	2298.18	429.42	2784.00	396.49	.41
im-pos cells	337.27	196.50	214.00	84.77	.57
im-neg cells	1960.90	381.22	2570.00	365.98	.26
LI	12.00	5.41	6.57	2.71	.38
Grade III					
all cells	2923.33	504.95	4232.22	365.09	.06
im-pos cells	521.66	222.95	405.77	175.80	.69
im-neg cells	2401.66	507.84	3624.44	522.52	.11
LI	18.38	9.62	17.05	6.94	.91
Grade IV					
all cells	3723.75	581.04	3529.33	487.31	.80
im-pos cells	976.25	315.50	401.33	159.00	.13
im-neg cells	2747.50	406.02	3128.00	515.60	.56
LI	23.87	6.65	12.70	4.80	

Discussion

So far, only few and rather controversial immunohistochemical studies describing the occurrence of iNOS in various brain tumors were published. The pattern of expression of the three NOS isoforms in primary CNS neoplasms including astrocytic tumors, meningiomas, schwannomas, ependymomas, medulloblastomas, and mixed gliomas was examined immunohistochemically [31]. Unfortunately, the authors mixed up the roman numbering of the three isoforms with their respective letter designations. Thus, endothelial NOS reads as II instead of III and inducible NOS reads as III instead of II. With regard to the inducible NOS isoforms, these authors obtained the following results: (1) iNOS immunoreactivity was less prevalent in the tumors and was usually confined to the tumor vasculature, (2) distinctively higher levels of iNOS were expressed in high grade astrocytic tumors compared to WHO grade II tumors and normal brain tissue, (3) iNOS immunoreactivity was rarely detectable in tumor cells, although moderate staining was seen in tumor endothelial cells, and (4) Western blots revealed no immunoreactivity in lysates of either normal brain or tumor [31].

Other results were published, reporting upregulation of iNOS in nearly 50% of gliomas, although only nNOS expression correlated with tumor grade [36]. The co-expression of NOS I-III was studied in 220 GBMs showing that all of the specimens revealed some NOS expression with NOS II expression in macrophages, microglia and endothelial cells, NOS III and I in GBM cells, and NOS III in

Table 5: Numerical density (n/mm²) of immunopositive (im-pos) cells and immunonegative (im-neg) cells and labeling index (%) in astrocytomas with malignant progression: Differences between time of operations (OP).

	OP 1		OP 2		
	Mean	Sem	Mean	Sem	P-value
all cells	2461.81	432.08	2906.36	307.15	.41
im-pos cells	480.00	211.24	562.72	191.51	.77
im-neg cells	1981.81	407.22	2343.63	259.48	.46
LI	18.19	6.90	16.75	5.44	.87
	OP 2		**OP 3**		
all cells	2906.36	307.15	4520.00	1599.82	.42
im-pos cells	562.72	191.51	1060.00	762.71	.58
im-neg cells	2343.63	259.48	3460.00	918.82	.34
LI	16.75	5.44	16.30	10.30	.97
	OP 1		**OP 3**		
all cells	2461.81	432.08	4520.00	1599.82	.32
im-pos cells	480.00	211.24	1060.00	762.71	.53
im-neg cells	1981.81	407.22	3460.00	918.82	.24
LI	18.19	6.90	16.30	10.30	.88
	OP 1		**OP 2+3**		
all cells	2461.81	432.08	3252.14	418.47	.20
im-pos cells	480.00	211.24	669.28	211.06	.53
im-neg cells	1981.81	407.22	2582.85	290.97	.24
LI	18.19	6.90	16.65	4.62	.85

endothelial cells [43]. Inducible NOS II in any expression grade was observed in 47.5% of the specimens. Significant correlations were observed for the expression of the macrophage marker Ki-MIP with NOS II, and VEGF-R1 with NOS II and NOS III [43]. Low numbers of macrophages/microglia expressing NOS II in GBMs were described; no information was provided as to whether tumor cells were stained [44].

Tumor cells expressing all three NOS isoforms in brain tumors except for GBM and metastatic adenocarcinoma were reported [45]. In four tumors, cells (lymphocytes and macrophages) were intensely labeled with MacNOS in and around the blood vessels. The authors concluded that nitric oxide is produced in the tumor cells and endothelium of tumor vasculature while occasionally glial cells may also produce it [45]. NOS and 5'-nucleotidase were observed in GBM tumor cells [46]. suggesting that both the cytotoxic effects due to NO production by tumor cells and the non-catalytic role of membrane 5' nucleotidase acting as an adhesive molecule favor tumor invasiveness [46]. The apparent association of increased iNOS immunoreactivity and NADPH-diaphorase activity with the histological grade of astrocytic tumors suggested that NO may be an important molecule in mediating pathological processes characteristic of highly malignant tumors. High levels of iNOS and NO production may influence brain tumor growth *in vivo* [47].

Glioma Stem Cells (GSCs) produce nitric oxide via high NOS2 expression which correlates with decreased survival in human glioma patients. NOS2 inhibition slows glioma growth in a murine intracranial model. Thus, NOS2 inhibition may be an efficacious approach to treating this devastating disease [48]. Reactive glial cells and the endothelium of small blood vessels displayed strong NADPH-d and iNOS activities in edematous peritumoral tissue [49]. In tumor cortex, NADPH-d and iNOS positive neurons were reduced in number and their dendrites were thin and interrupted, and infiltrates of NADPH-d and iNOS-positive tumor cells were frequent. Expression of eNOS in tumor vessels was significantly correlated with histological grade and proliferative potential [50]. Induction of VEGF gene expression in human A-172 GBM cells by NO is mediated through guanylate cyclase activity and requires on-going protein synthesis [51]. NO generated by iNOS expression inhibits hypoxia-inducible factor-1 (HIF-1) activity in hypoxic C6 glioma cells reveals a negative feedback loop in the HIV-1 → iNOS cascade [52]. As a matter of fact, the authors showed increased NO synthesis by cytokine exposure or iNOS overexpression neutralized the cytotoxicity of BCNU and CCNU, but not cisplatin in rat C6 glioma cells. Expression of iNOS inhibited HIF-1 activity under hypoxia in C6 glioma cells transfected with a VEGF promotor-driven luciferase gene. Pretreatment of C6 cells with the antioxidant N-acetyl-1-cysteine, mollified the inhibitory effect of iNOS on HIF-1 binding. HIF-1 is known to be present at high levels in human tumors and that it is crucial in tumor promotion by up-regulating several target genes. HIF-1 stimulates the production of NO through the induction of inducible NO synthase (iNOS). HIF-1alpha and iNOS expressions did not correlate with patient survival [53].

By investigating immunohistochemical expression of COX-2, iNOS, and VEGF in 51 high-grade astrocytomas the relationship with microvessel density and prognostic significance were determined. Stepwise increase of immunoreactive scores for COX-2, iNOS, and VEGF was found from astrogliosis, through low-grade to high-grade astrocytoma. The COX-2 expression strongly correlated with iNOS, VEGF, and high MVD, both overall and in all tumors, whereas iNOS expression was weakly associated with VEGF and high MVD. Univariate analysis revealed a significant association between COX-2 overexpression and a poor outcome [54].

To verify the hypothesis that NO is involved in p53-dependent response to many kinds of stress, such as heat shock and changes in cellular metabolism, the effect of NO produced endogenously by heat-shocked cells on nonstressed cells using a human glioblastoma cell line, A-172, and its mutant p53 (mp53) transfectant (A-172/mp53) was examined [55]. The accumulation of iNOS was caused by heat treatment of the mtp53 cells but not of the wild-type p53 (wtp53) cells. The accumulation of heat shock protein 72 (hsp72) and p53 was observed in nontreated mtp53 cells cocultivated with heated mp53 cells, and the accumulation of these proteins was suppressed by the addition of a specific iNOS inhibitor, aminoguanidine, to the medium. Furthermore, the accumulation of these proteins was observed in the wtp53 cells after exposure to the conditioned medium by preculture of the heated mp53 cells, and the accumulation was completely blocked by the addition of a specific NO scavenger, 2-(4-carboxyphenyl)- 4,4,5,5-tetramethyl-imidazoline-1- oxyl-3-oxide, to the medium. The accumulation of hsp72 and p53 in NO-recipient cells cocultivated with heated NO-donor cells provides the first evidence for an intercellular signal transduction pathway via NO as intermediate without cell-to-cell interactions such as gap junctions. Furthermore, modifications of p53 in gliomas *in vivo* could be mediated by peroxynitrite, a highly reactive metabolite of NO [47]. Accumulation of iNOS was caused by X irradiation of mutant TP53 but not of the wild-type TP53 glioblastoma cells [55].

Several pathways might be involved in the patheogenic repertoire of NO. There is evidence for a regulatory axis of high grade glioma

cell movement from NO through MMP-1, with NOS inhibitor results showing promise for future pharmacologic investigation [56]. Intracellular NO affects IRE1-alpha-dependent CREB phosphorylation in human glioma cells. Therefore, an IRE1-alpha-dependent phospho-CREB signaling pathway responsive to NO/Ca(2+) may play an important role in regulating ER-related cell death in glioma [57]. Results have been reported indicating that the NO/Ca2þ/CaM/ERK signalling pathway is a mechanism mediating the mitogenic effect of IL-1b in human astrocytes. A nitric oxide/Ca2þ/calmodulin/ERK1/2 mitogenactivated protein kinase pathway is involved in the mitogenic effect of IL-1ß in human astrocytoma cells [77]. It was observed that the stimulatory effect of 5-AzadC on iNOS expression was independent of DNA demethylation. α9β1 integrin-mediated cell migration utilizes the iNOS pathway, and inhibition of the migratory potential of glioma cells by simultaneous knockdown of MMP-9 and uPAR could be attributed to the reduced α9β1 integrin and iNOS levels [59].

iNOS levels in GBM were positively correlated with IL-1ß mRNA, but not with other cytokines like TNF-alpha, interferon-gamma, TGF ß1 and TGF ß2 [60]. The survival duration was enhanced when levels of IL-1ß mRNA were elevated or when levels of TGFß2 were low, but was independent of the level of iNOS mRNA within the tumor [60]. Incubation of C6 astrocytoma cells with bacterial endotoxin (lipopolysaccharide; LPS) plus interferon-gamma (IFN-γ), or with a combination of cytokines (TNF-α, IL-1ß, and IFN-γ) leads to high levels of iNOS expression [61]. It was concluded that astrocytoma cells possess a cytokine-inducible Ca(++)-calmodulin-independent NO-synthase, whose activation seems to occur with a mechanism different from that described for LPS [62]. Lipopolysaccharide and interferon-γ, which are able to strongly induce iNOS in astrocytoma cells, can rapidly inhibit the NO production generated by the constitutive NOS isoform [63]. Thus, the results suggest a possible role of a constitutive NOS isoform in astrocytes as a control function on iNOS induction [63]. Data show that IFN-γ increases the synthesis and release of NO by cultured astrocytoma cells and this could co-participate in the MHC II antigen expression by this cell type [64].

Several papers were published, in which the production of NO by brain tumor cells and the influence of possible regulatory factors was studied in cell culture systems. RhoA was identified as a negative regulator of iNOS expression via the inactivation of NF-kappaB in transformed brain cell lines C(6) glioma, human astrocytoma (T98G, A172), neuroblastoma (NEB), and immortal rat astrocytes. It was reported that downregulation of RhoA by lovastatin resulted in increased iNOS expression via the activation of NF-kappaB-CBP/p300 pathway in transformed brain cells [9,65-67]. It has been observed that conditioned medium from activated microglia resulted in the induction of iNOS in C6 cells, and IL-1ß was shown to be a key regulator of iNOS induction [68-70].

Some therapeutically used agents were described to potentiate the iNOS activity. As astrocytes could modulate CNS autoimmunity iNOS-mediated production of immunoregulatory free radical NO, the effect of taxol, a microtubule-stabilizing agent, on NO synthesis has been investigated in rat astrocytes. Taxol, either alone or in combination with interferon-*γ*, induced NO generation in primary astrocytes and astrocytoma C6 cells in a dose- and time-dependent manner NO release by taxol-stimulated astrocytes was blocked with the microtubule-depolymerizing agent colchicine [71]. Although a heterogeneous nitric oxide production by xenografted glioma cells may impact VEGF-A and Cyclin D1 expression levels, a reduction of nitric oxide levels by nitric oxide scavenging could be an efficient approach to treat glioma [72]. The reciprocal activation of glioma cells and microglia via ROS-dependent iNOS/NO elevation at least partially mediated by TNF-α and MCP-1 was shown by [73]. L-NMMA and aminoguanidine, competitive inhibitors of iNOS, suppressed NO production as measured by the NO by-product, nitrite, as did IFN-ß. Dexamethasone enhanced the NO production, and IFN-ß decreased the amount of the enhancement. Neither IL-10 nor TGF-ß inhibited nitrite production. High levels of NO suppress IL-8 production by T98G GBM cells, and murine IL-1 alpha plays a major role in the induction of IL-8 production by these T98G cells [74]. It is, therefore, possible that excessive production of NO during the interaction of glioma cells with macrophages may play a regulatory role in chemokine production, thus mitigating inflammatory responses. IL-17 caused a dose-dependent enhancement of IFN-γ-triggered NO synthesis in both mouse and rat primary astrocytes [75]. IFN-γ-triggered expression of mRNA for iNOS, but not for its transcription factor interferon regulatory factor-1, which was markedly elevated in IL-17-treated astrocytes.

Massive production of NO by iNOS has been shown to exert tumoricidal effects. However, NO may enhance vasodilation and promote neovascularization, thereby facilitating tumor growth. Compared to the effects of NO on tumor cell death and survival, correlation between NO and cytotoxicity of chemotherapeutic reagents in glioma have been less well characterized. HIF-1 contributes to iNOS induction under hypoxia. It was reported that that increased NO synthesis by cytokine exposure or iNOS overexpression neutralized the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), but not cisplatin, in rat C6 glioma cells. That NO generated by iNOS expression inhibits HIF-1 activity in hypoxic C6 cells reveals a negative feedback loop in the HIF-1 → iNOS cascade [52]. Oral administration of L-arginine or hydroxyurea selectively increased tumor permeability, which is likely mediated by alteration in cGMP levels. The findings of the authors suggest that use of oral NO donors may be a strategy to enhance the delivery of chemotherapeutics to malignant brain tumors [76].

Tumors prone to edema formation, such as high grade astrocytomas, were more likely to have higher levels of NOS II and NOS III reactivity than tumors which are not characterized by edema formation, such as juvenile pilocytic astrocytomas or schwannomas [31]. Thus, NO produced by these tumor capillaries is likely to contribute to edema formation, and one strategy for selectively reducing tumor blood flow and edema might involve inhibition of NO production. Interestingly, dexamethasone, a corticosteroid used to treat increased intracranial pressure due to brain tumor edema, is a well-known inhibitor of NOS III but not of the constitutive NOS I and NOS II isoforms [77]. Also, the presence of NOS II and NOS I in tumor endothelial cells, often at high levels, suggests that other inhibitors of NOS which block NO production by these NOS isoforms may be effective in further reducing tumor edema and blood supply to the tumor by selectively blocking NOS activity in the tumor cells and tumor endothelial cells.

NO plays an important role in recent conceptions of tumor pathogenesis, although the biologic role NO plays in malignancies is still unclear. The supraphysiologic production of NO in malignant tissue by iNOS may cause cytotoxicity, and supports as inhibits

immune defense mechanisms as described [78]. Furthermore, NO may increase tumor blood flow and promote angiogenesis with NO having both pro- and antitumor-actions depending on its concentration As seen in the present study, iNOS expression in tumor cells is highly inhomogeneous and, hence, the role of NO must be quite different with regard to the situation within the NO-producing cell and transcellular effects on surrounding tissue.

NO may be responsible for increasing blood flow to tumors expressing high levels of NOS [4]. Neuronal NO regulates cerebral blood flow in the normal brain, and endothelial-derived NO results in vasodilatation of normal blood vessels and inhibits platelet aggregation [79]. NO has been identified as an important regulator of tumor blood flow in experimental tumors in mice, and inhibition of NO production in these tumors by systemically administered NOS inhibitors decreased tumor blood flow and reduced tumor growth [80]. Rapidly growing tumors such as GBMs are highly vascular and have altered blood flow dynamics [81]. NO production by tumor cells and tumor endothelial cells may play, therefore, a critical role in ensuring maximum blood flow to the tumor cells. NO production by capillary endothelial cells influences the degree of vascular permeability in blood vessels, and NOS inhibitors can decrease local edema formation by experimental tumors in mice [5].

In summary, nitric oxide has both physiological roles (e.g., neurotransmitter-like activity, stimulation of cyclic GMP), and pathophysiological roles (e.g., neoplastic transformation, tumor neovascularization, induction of apoptosis, free radical damage). Moreover, whether nitric oxide is neuroprotective or neurotoxic in a given disease state, or whether it enhances or diminishes chemotherapeutic efficacy in malignant neoplasia, is unresolved [82].

Emerging knowledge of the dual and diverging role of nitric oxide in glioma biology has focused on possibilities to achieve anti-glioma effects by modulation of Nitric Oxide (NO) release and function in these tumors. NO has been shown to influence proliferation of glioma cells, vascular function in gliomas, invasive capacity of gliomas, effects of chemo and radiotherapy and also immune reactivity against these tumors. The mechanisms behind the reported diverse and dual effects of NO in glioma biology are multiple. Some of the diversity can be explained by different experimental setups as *in vitro* versus *in vivo* models but the cellular sources, timing, absolute levels and gradients play a decisive role for the effects of NO on glioma biology. Current research in this field is hampered by the lack of inhibitors and donors approved for clinical use [83].

In the present study, the expression of the inducible isoform of the NO producing enzyme, iNOS, could be distinctly demonstrated in astrocytomas of various grades of malignancy. The immunohistochemical iNOS reactions did not show a clear correlation with tumor grade or survival. Further investigations of the pathophysiologic and protective roles of NO and the influence of NO inhibitors on tumor growth in brain tumors will be necessary to elucidate the role of iNOS in astrocytomas and to see how and to what extent they are functionally interrelated. NO plays an intriguing, controversial and complicated role in tumor growth, survival and invasion.

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