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Advances in Brief

Expression of Nitric Oxide Synthase in Human Central Nervous System Tumors[1]

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Abstract

The nitric oxide synthases (NOS) are a family of related enzymes which regulate the production of NO, a free radical gas implicated in a wide variety of biological processes. Vasodilation and increased tumor blood flow, increased vascular permeability, modulation of host tumoricidal activity, and free radical injury to tumor cells and adjacent normal tissues are pathophysiological features of malignant tumors that may be mediated by NO. We examined human brain tumors for three NOS isoforms and NADPH diaphorase, a histochemical marker of NOS activity in the brain. We detected increased expression of the brain and endothelial forms of NOS [NOS I and NOS II, respectively (C. Nathan and Q. Xie. Cell, *78:* 915–919, 1994)] in astrocytic tumors, and the highest levels of expression was found in higher grade tumors. Each of these two isoforms was found in tumor cells and tumor endothelial cells. The macrophage isoform of NOS (NOS III) 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. Western blot analysis of tumor tissues for these NOS isoforms confirmed these observations. Our data indicate that malignant central nervous system neoplasms express unexpectedly high levels of NOS and suggest that NO production may be associated with pathophysiological processes important to these tumors.

Introduction

NO is an important molecule in multiple signal transduction pathways and is synthesized by a family of three distinctive NOS[3] isoforms named for the tissues in which they were originally described (1, 2). NOS I and NOS II are calcium dependent and constitutively expressed in a variety of cells including subpopulations of neurons and endothelial cells, respectively. An inducible calcium independent form, NOS III, may be found in macrophages, 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). NOS activity has been documented in cell lines derived from several tumor types, but to date NOS expression has been identified only in pathological specimens of human ovarian cancer (4).

Several pathophysiological properties important for tumor cell survival and tumor pathology may be mediated by NO. Recent studies have suggested a role for NO in causing increased tumor blood flow, edema, and vascular permeability (5–7). 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, and γ-interferon induce NOS activity *in vitro* (8). Because of the possible role of NO in the pathophysiology of brain tumors, we evaluated specimens of human CNS tumors for NOS expression by immunohistochemistry, NADPHd histochemistry, and Western blot analysis.

Materials and Methods

Materials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Monoclonal antibodies for brain, endothelial, and macrophage NOS were obtained from Transduction Laboratories (Louisville, KY).

Tissue Collection and Histological Evaluation. Pathological specimens from 36 different brain tumor patients were included in this study. Frozen specimens of brain tumors were obtained from the UCSF Brain Tumor Research Center tumor bank. These frozen tissues were obtained at the time of surgery and placed immediately in liquid nitrogen and stored at −70°C until prepared for evaluation. Paraffin blocks of brain tumor and normal brain tissue were obtained from the UCSF Department of Pathology. Tumor type and grade were determined by histological assessment by UCSF neuropathologists according to the WHO criteria for the grading of brain tumors.

NADPH Diaphorase Staining. To evaluate NADPHd, 10-μm-thick frozen sections were fixed for 1 h at 4°C in PBS containing 4% paraformaldehyde. Sections were then washed in 100 mM Tris-HCl, pH 7.4, for 5 min at room temperature and incubated in 100 mM Tris-HCl, pH 7.4, containing 1 mM NADPH, 0.3 mM nitro blue tetrazolium, and 0.2% Triton X-100 at room temperature for 30 min. These histological sections were then dehydrated through graded alcohols and xylene and mounted for microscopic examination.

Immunohistochemical Analysis of Tissue Sections. Histological sections (6 μm) of paraffin wax-embedded, fixed tissues were dewaxed in xylene and hydrated through graded alcohols to PBS. After a wash in PBS/Tween, endogenous peroxidases were blocked by incubation in PBS containing 3% hydrogen peroxide and 0.2% Tween 20 at room temperature for 15 min. The sections to be stained for NOS III and the controls for NOS III were washed in PBS/Tween for 5 min and then boiled in 10 mM citrate buffer, pH 6.0, for 10 min to enhance antigen retrieval. After further washing in PBS/Tween, the sections were blocked and immunolabeled. For NOS detection, the samples were incubated with mouse monoclonal antibodies against NOS I, NOS II, or NOS III, diluted 1:250, 1:100, or 1:50, respectively, in 10% normal rabbit serum. After incubation overnight at 4°C these sections were washed in PBS/Tween (three times for 2 min each) and incubated with rabbit anti-mouse biotinylated IgG (Zymed Laboratories, South San Francisco, CA). Mouse IgG was used at identical concentrations as a control primary antibody. Biotinylated conjugates were detected with avidin-peroxidase conjugate (Zymed Laboratories). Immunolabeling was detected with the chromogen diaminobenzadine tetrahydrochloride, after which the slides were washed in water, stained with hematoxylin, dehydrated, and mounted for examination.

The NOS immunoreactivity and NADPHd activity of all specimens were evaluated blindly by two independent observers. A grade of 0 was assigned to tumors with no detectable signal and grades of 1, 2, and 3 were assigned to tumors with light, moderate, and intense reactivity, respectively. Normal brain tissues, when only the expected staining of rare neurons was observed, were graded as 0.

Western Blotting. Protein homogenates from human brain tumor specimens or normal mouse brain were prepared in 10 volumes of buffer A (25 mM Tris-HCl, pH 7.4–100 mM NaCl-1 mM EDTA-1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid-1 mM phenylmethylsulfonyl fluoride). Following

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[3] The abbreviations used are: NOS, nitric oxide synthase; CNS, central nervous system; UCSF, University of California at San Francisco; NADPHd, NADPH diaphorase; PBS/Tween, PBS containing 0.05% Tween 20.

centrifugation at 15,000 rpm for 20 min soluble extracts were partially purified using 2′,5′-ADP agarose chromatography as described previously (9). Control protein lysates for NOS II and NOS III were provided by Transduction Laboratories. Mouse brain lysate was used as a control for NOS I activity. Affinity-purified samples were fractionated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with antibodies (anti-NOS 1 antibody, 1:250 dilution; anti-NOS II and anti-NOS III antibodies, 1:500 dilution). Immunoreactive species were visualized by enhanced chemiluminescence (Amersham).

Results

We used immunohistochemical analysis to examine the pattern of expression of NOS isoforms in primary CNS neoplasms. High levels of NOS immunoreactivity were detected in a variety of malignant brain tumors (Table 1). We detected NOS I expression in most tumor specimens examined (Table 1), despite the diverse cell types in which these tumors arise. NOS II expression occurred frequently in both tumor vascular tissue and tumor cells (see below). NOS III immunoreactivity was less prevalent in the tumors and was usually confined to the tumor vasculature. The highest levels of NOS I and NOS II immunoreactivity were found in high grade gliomas, in some juvenile pilocytic astrocytomas, in medulloblastomas, and in a mixed malignant glioma (Table 1). Of interest in this regard is the distinctively higher level of NOS expression in high grade astrocytic tumors compared to WHO Grade II tumors and normal brain tissue. Immunohistochemical analysis of normal brain revealed NOS I reactivity only in rare neurons, while NOS II and NOS III were not detected (data not shown).

Table 1 *NOS expression in CNS tumors*

Tumor type	NOS I	NOS II	NOS III	NADPHd
WHO Grade IV astrocytoma (glioblastoma)	3+	3+	0	
	3+	3+	0	
	3+	1+	0	3+
	1+	1+	1+	3+
	3+	3+	1+	
	3+	2+	2+	
	3+	3+	0	3+
	2+	2+	0	
	3+	2+	1+	2+
WHO Grade III astrocytoma (highly anaplastic astrocytoma)	2+	0	0	3+
	2+	2+	1+	3+
	2+	2+	0	1+
	3+	2+	0	
	2+	3+	3+	
	3+	3+	1+	
	3+	0	0	
WHO Grade II astrocytoma (moderately anaplastic astrocytoma)	0	0	0	
	2+	0	0	
	0	0	0	1+
	1+	1+	0	1+
Juvenile pilocytic astrocytoma	1+	0	0	2+
	1+	0	0	
	2+	2+	0	2+
	2+	0	0	
	3+	0	0	
Meningioma	1+	0	0	3+
	0	1+	0	1+
Aggressive meningioma	0	0	0	
	2+	2+	0	
Schwannoma	0	0	0	
	0	1+	0	1+
	1+	0	0	
Ependymoma	2+	2+	0	
Medulloblastoma	2+	2+	0	
	3+	3+	0	
Mixed glioma	3+	2+	1+	
Gliosis	0	0	0	
	0	3+	0	0
Normal adult brain[a]	0	0	0	0
	0	0	0	0
	0	0	0	
	0	0	0	

[a] The normal pattern of isolated neuron staining for NOS I and NADPHd was seen.

Glioblastomas, which are characterized by rapid growth, vascular proliferation, and edema, expressed readily detectable NOS I and NOS II (Table 1). Adjacent sections of a representative glioblastoma shown in Fig. 1, *A-D*, illustrate marked tumor cell immunoreactivity with both NOS I and NOS II and intense NOS II reactivity of endothelial cells within the tumor vasculature (Fig. 1, *B* and *C*). NOS III immunoreactivity was rarely detectable in tumor cells, although moderate staining was seen in tumor endothelial cells (Fig. 1*D*). Examination of these specimens at higher magnification revealed that cells which were strongly immunoreactive for NOS I and NOS II were often adjacent to cells with no reactivity (data not shown). Also, immunoreactivity for these two isoforms was often found in the cytoplasm and in the nucleus of the tumor cells. We observed that cells with immunoreactive nuclei were often characterized by their high nuclear to cytoplasmic ratio and prominent nucleoli (data not shown).

These immunohistochemical findings were confirmed by Western blot analysis of several tumor specimen lysates none of which had been examined by immunohistochemistry (see "Materials and Methods"). Both normal brain and tumor tissues contained immunoreactive proteins of the appropriate size for NOS I (Fig. 2). We found higher levels of NOS I in tumors in which NOS I was readily detectable by immunohistochemistry (Fig. 2 and data not shown). Interestingly, many of the tumors examined by Western blot analysis for NOS I had a highly expressed, second immunoreactive species of greater mobility than previously described species. Western blot analysis of NOS II revealed no expression in normal brain, but we did identify NOS II in each of the tumors we examined (Fig. 2). Tumor specimens which were analyzed by Western blot and immunohistochemistry had comparable levels of NOS II immunoreactivity as assessed by both assays. Western blots of NOS III revealed no immunoreactivity in lysates of either normal brain or tumor (Fig. 2 and data not shown).

To extend our finding of increased NOS expression, we examined frozen tumor specimens for NADPHd activity, since NADPHd activity correlates with NOS activity in the brain (2). Thirty-five tumor specimens were analyzed for NADPHd histochemistry, and those which were also examined for immunohistochemistry are shown in Table 1. We found that all tumor specimens had increased NADPHd activity as compared to normal brain controls (Table 1 and data not shown). NADPHd was readily detectable in malignant brain tumors (Table 1, Fig. 3),[4] providing evidence of NOS activity in CNS tumors. In Fig. 3, we demonstrate two areas of the same Grade III astrocytoma with different histological characteristics. In an area of the tumor section with histological characteristics indistinguishable from those of normal brain tissue (Fig. 3*A*), NADPHd activity is found only in an isolated neuron (Fig. 3*B*). However, in a region containing a high proportion of malignant cells (Fig. 3*C*), NADPHd staining was observed in numerous astrocytic tumor cells (Fig. 3*D*). Also shown in Fig. 3, which is representative of the pattern of NADPHd staining seen in other malignant astrocytic tumors, is intense NADPHd staining of vascular structures within the tumor. This contrasts sharply to a lack of NADPHd activity in blood vessels of normal tissue

[4] Unpublished data.

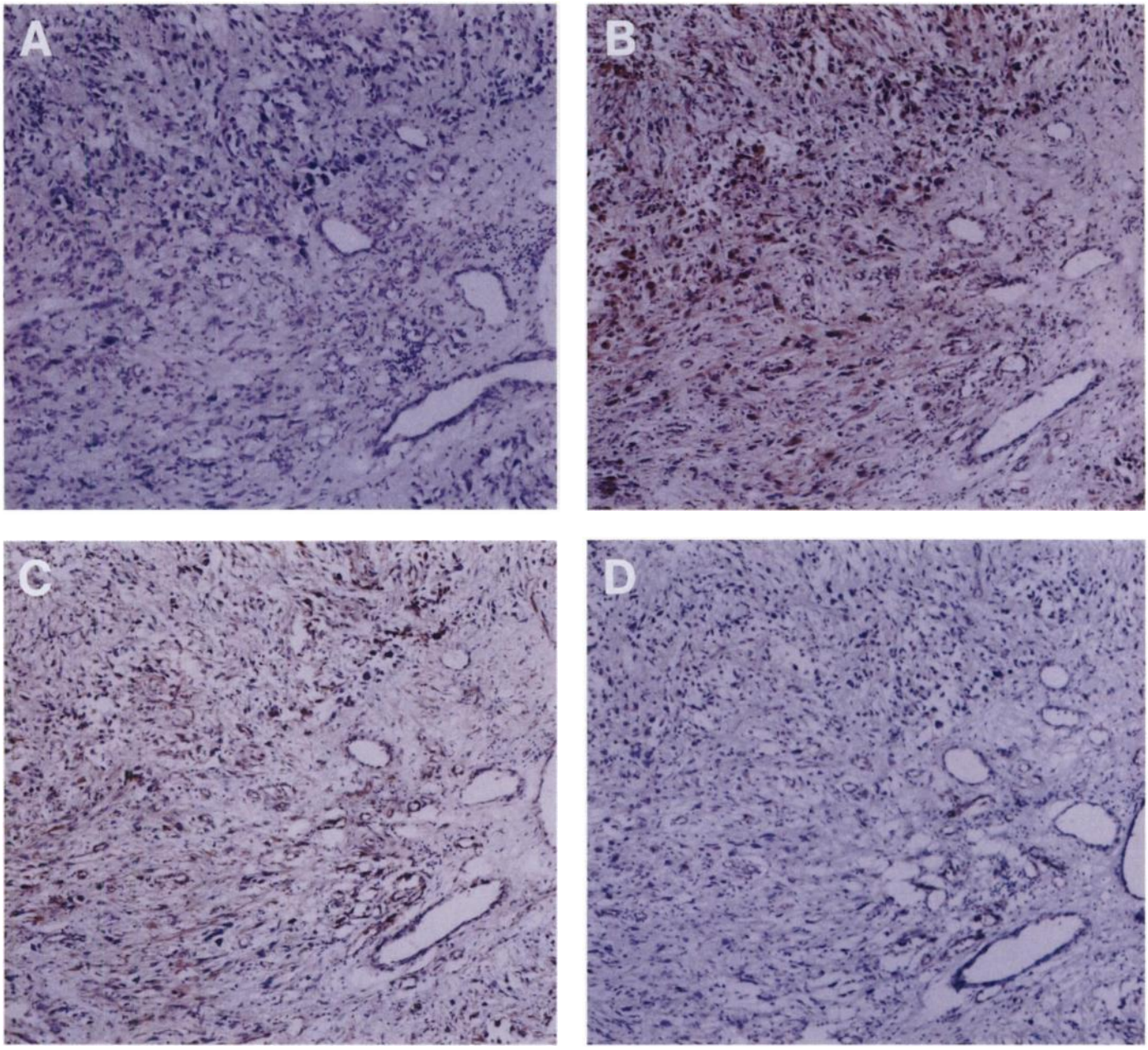

Fig. 1. Evaluation of NOS expression in a Grade IV astrocytoma (glioblastoma). Sequential histological sections were examined immunohistochemically with a control antibody (*A*), or antibodies against NOS I (*B*), NOS II (*C*), or NOS III (*D*).

(data not shown) or less cellular tumor regions (Fig. 3*B*). Glioblastomas and high grade astrocytomas generally had moderate to intense NADPHd staining, while normal brain had only rare neuronal staining. In almost every case in which NADPH staining was detected, NOS immunoreactivity was present at increased levels (Table 1).[4]

Discussion

We found NOS I and NOS II, which have been traditionally considered constitutive and noninducible, to be highly expressed in several different CNS tumors. NOS III, which is calcium independent and inducible, was rarely expressed in these tumors. This observation that NOS and NADPHd are present at increased levels in primary CNS neoplasms implicates NO in the pathophysiology of these tumors. The apparent association of increased NOS immunoreactivity and NADPHd activity with the histological grade of astrocytic tumors suggests that NO may be an important molecule in mediating pathological processes characteristic of highly malignant tumors which distinguish them from lower grade tumors.

High levels of NOS and NO production may influence brain tumor growth *in vivo*. Induction of NOS by tumor necrosis factor α and γ-interferon is associated with an increased growth rate of rat C6 glioma cells *in vitro*, and this effect can be diminished by an inhibitor of NOS (10). Although the mechanism by which NO may influence glial tumor cell growth is not known, it is possible that NO may act in signal transduction pathways of these cells modifying the expression of genes involved in cellular proliferation (11). It is also known that neurons expressing NOS in the brain are resistant to excitotoxic cell death in stroke and are more resistant to cell death in neurodegenerative diseases such as Huntington's and Alzheimer's disease (12). Thus tumor cells which produce NO may themselves have a

survival advantage *in vivo* similar to that seen in neurons which express NOS.

NO may be responsible for increasing blood flow to tumors expressing high levels of NOS (5). Neuronal NO regulates cerebral blood flow in the normal brain, and endothelial-derived NO results in vasodilation of normal blood vessels and inhibits platelet aggregation (13). NO has recently 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 (14, 15). Rapidly growing tumors such as glioblastomas are highly vascular and have altered blood flow dynamics (16). NO production by tumor cells and tumor endothelial cells may play 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 (6). Our data show that NOS immunoreactivity is often greatly increased in tumor vasculature (Figs. 1 and 3) and evaluation at higher magnification localized this reactivity to brain tumor capillary endothelial cells, while it is not detectable in normal brain endothelial cells (data not shown). Furthermore, we found that 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. 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 (17). Although NOS III was less immunoreactive in tumors than the other isoforms of NOS, it was expressed in the vasculature of high grade gliomas. The inhibition of NOS III may contribute to the therapeutic effects seen in brain tumor patients treated with dexamethasone. Also, our observation 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.

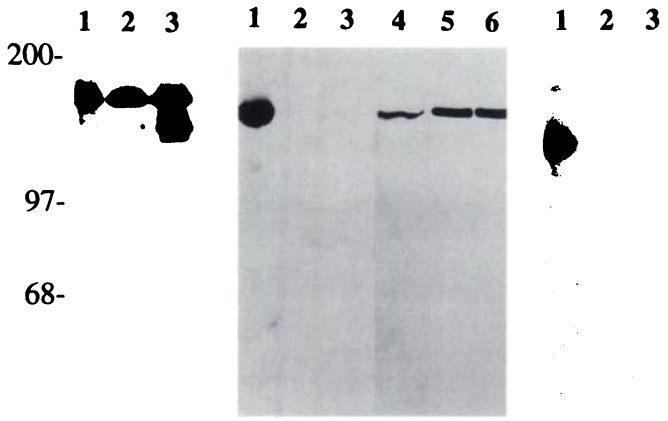

Fig. 2. Western blot analysis of normal and malignant CNS tissues for NOS. *Lane 1* in each panel contains protein lysate of cells known to express the isoform being examined. Antibodies reactive against NOS I (left panel), NOS II (middle panel), and NOS III (right panel) were used to examine lysates of normal brain (*Lane 2* in each panel) and a juvenile pilocytic astrocytoma (*Lane 3* in each panel). In the middle panel, which examines NOS II reactivity, the lysates of a glioblastoma (*Lane 4*), an ependymoma (*Lane 5*), and a malignant meningioma (*Lane 6*) are also shown.

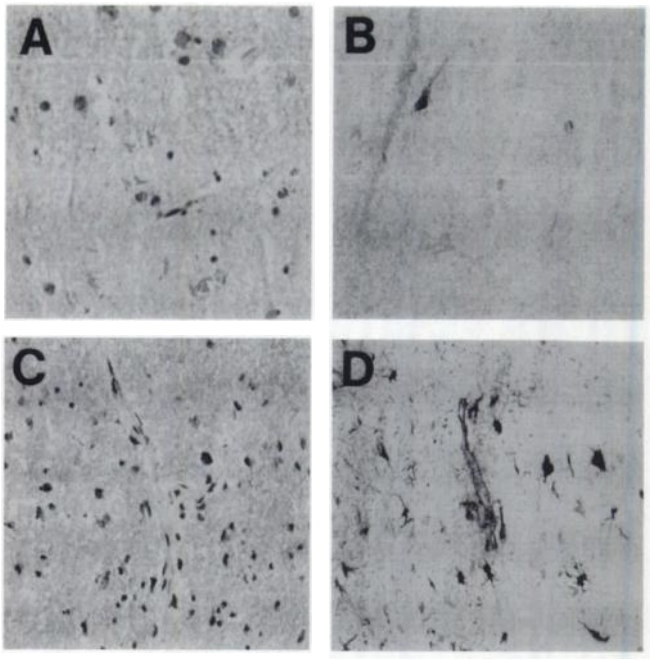

Fig. 3. Histochemical evaluation of NADPHd activity in a Grade III astrocytoma. Regions of the tumor appearing normal (*A*) and malignant (*C*) are shown next to sequential sections of the same region evaluated for NADPHd (*B* and *D*, respectively). H & E.

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