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Overexpression and localization of cathepsin B during the progression of human gliomas

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Degradation of the extracellular matrix is a prerequisite for acquisition of the invasive phenotype. Several proteinases released by invading tumor cells appear to participate in the focal degradation of extracellular matrix proteins. Using an enzyme-linked immunosorbent assay, enzymatic assays, Western and Nothern blotting techniques, we determined whether increased levels of the cysteine protease cathepsin B correlated with the progression and invasion of human gliomas. The amount of cathepsin B activity and protein content were highest in glioblastomas, lower in anaplastic astrocytomas and lowest in normal brain tissue and low-grade gliomas. There were significantly higher amounts of *Mr* 25000 and 26000 bands in glioblastoma and anaplastic astrocytoma than in normal brain and low-grade glioma tissue extracts as determined by Western blotting with anti-cathepsin antibodies. In addition, cathepsin B transcripts were overexpressed in anaplastic astrocytoma (about two- to three-fold), in glioblastoma (about eight- to 10-fold), compared with normal brain tissue and low-grade glioma. Immunohistochemical staining for cathepsin B showed intense immunoreactivity in tumor and endothelial cells of glioblastomas and anaplastic astrocytomas but only weak immunoreactivity in low-grade glioma and normal brain tissues. Therefore, we conclude that cathepsin B expression is greatest in highly malignant astrocytomas, especially in glioblastomas, and is correlated with the malignant progression of astrocytomas.

Keywords: cysteine proteases, extracellular matrix, glioblastoma multiforme, invasion

Introduction

The invasiveness and destructive properties of malignant neoplasms in the central nervous system (CNS) vary between different types of tumor. Higher grade tumors, such as glioblastomas, have a poor prognosis with a mean survival of 8 to 12 months after chemotherapy and/or irradiation [1]. The poor prognosis of CNS tumors is due, in part, to the difficulty of accomplishing a total resection because of diffuse infiltrative growth into the adjacent brain tissues [2], and to residual tumor cell resistance to irradiation [3], and cytostasis [4]. Thus recurrence at the site of the initial lesion occurs often. Immunohistochemical examination of the glial limitans externa has shown that it contains interstitial collagen, fibronectin, laminin, and type IV collagen [5]. The invasion of many primary brain tumors is thought to be accompanied by elevations in the levels of proteinases. This allows breaching of connective tissue extra-cellular matrix (ECM) barriers remodeling of vasculature ECM and destruction of normal brain tissue.

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The expression and secretion of proteolytic enzymes such as collagenases, cathepsins, plasminogen activators, and plasmin have been implicated in tumor invasion and metastasis formation [6]. Cathepsin B, a cysteine proteinase, has been reported to be an important degradative enzyme in invasion and metastasis [7]. Cathepsin B is expressed at higher levels in invasive tumors than in normal or benign tissues. It is thought to play a regulatory role in collagen degradation because it can convert inactive procollagenase type IV to its active form [8] and efficiently convert soluble or tumor-cell-receptor-bound proenzyme urokinase type plasminogen activator (uPA) to an enzymatically active two-chain uPA [9]. Intracellular activity and secretion of cathepsin B has been described in a number of non-CNS human tumors, including malignant and nonmalignant breast tumors [10] and colonic adenocarcinomas [11]. Human glioma cell lines were recently reported to secrete cathepsin B *in vitro* [12]. However, the presence of cathepsin B in normal brain tissue or in primary brain tumors has not been reported. In the present study, we demonstrate the expression of cathepsin B enzyme activity and protein in normal brain tissue and primary brain tumors. The progression of human gliomas was associated with significantly increased levels of cathepsin B.

Materials and methods

Materials

Cathepsin B and rabbit anti-cathepsin B antibody were purchased from Athens Research and Technology Inc. (Athens, GA). Nα-CBZ-Arg–Arg–4-methoxy–β-naphthalamide, β-naphthalamine, L-*trans*-epoxy–succinyl–leucylamido(4-guanidino)butane (E-64), cysteine, fast garnet, mersalyl acid and peroxidase-conjugated goat anti-rabbit IgG were purchased from Sigma Chemical Co. (St Louis, MO). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). α-[^{32}P]-dCTP was purchased from DuPont NEN Research Products (Boston, MA). All other chemicals were of analytical grade.

Surgical specimens

Human brain tumor tissue and normal brain tissue samples were obtained from patients undergoing craniotomy to remove brain tumor. The samples were flash-frozen in liquid nitrogen immediately after surgical removal and stored at −80°C. Tissue samples for immunohistochemical analysis of cathepsin B were provided by the Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas and were fixed in 10% formalin and embedded in paraffin. The histological diagnosis was confirmed for each tissue block by standard light-microscopical evaluation of sections stained with hematoxylin and eosin. The samples included tissues from seven glioblastomas, five anaplastic astrocytomas, five low-grade astrocytomas, and five normal brains.

Preparation of tissue

Frozen normal brain and tumor tissues were thawed, homogenized in 50 mM acetate buffer (pH 5.2, with 0.1 M NaCl, 1 mM EDTA) containing 0.2% Triton X-100 on ice, and centrifuged at 10 000 *g* at −10°C for 30 min. The pellets were discarded and the supernatants aliquoted. Some of the aliquots were taken to determine total protein content [13].

Cathepsin B assay

Cathepsin B activity was determined in tissue extracts as described previously [14]. Normal brain tissue and tumor tissue extracts (50 μg) were incubated with activation buffer (88 mM KH_2 PO4, 12 mM Na_2 HP04, 1.33 mM disodium EDTA, pH 6.0, and freshly prepared 2.7 mM cysteine) at 37°C for 10 min. The reaction was initiated by adding 10 μl of 10 mM substrate (Nα–CBZ–Arg–Arg–4–methoxy–β-naphthalamide) and incubated at 37°C for 15 min. The enzymatic reaction was stopped by addition of 200 μl of coupling reagent (mersalyl-briJ-Fast garnet reagent) and the samples were incubated for 10 min for color development. Absorbance (540 nm) was determined for each sample. Controls were prepared by adding the enzyme after the color reagent. Standards were prepared by replacing the enzyme with 10–50 μl of 10 mM β-naphthalamine. Cathepsin B activity was expressed as nmoles of naphthalamide released per min per milligram of protein. To confirm that the measured activities were indeed caused by cysteine proteinases, we used the active-site inhibitor E-64 as a control to block the cathepsin B activity.

Western blotting

Normal brain tissues and brain tumor tissue extracts (50 μg) were electrophoresed on a 12% SDS-polyacrylamide gel, followed by transfer of the proteins to nitrocellulose paper, according to the method of Towbin *et al.* [15]. The nitrocellulose paper was then incubated in blocking buffer (1.5% bovine serum albumin, 0.15 M NaCl, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Tris-HCl, pH 7.6) for 6 h at room temperature and washed with antibody buffer (0.3% bovine serum albumin, 0.15 M NaCl, 20 mM Tris-HCl pH 7.6) 3 times for 10-min each. The strips were

incubated with rabbit cathepsin B/antibody (1:500 dilution) at 4°C overnight or at room temperature for 2 h; washed as described; incubated with a second antibody (goat anti-rabbit IgG peroxidase conjugate, 1:1000) for 2 h at room temperature; washed with Tris-HCl buffer as described; incubated with the substrate 2, 4 chloronaphthol and kept in the dark for 15–30 min for color development.

ELISA

Quantitative analysis of the content of cathepsin B in normal brain tissue and tumor tissue extracts (75 μg) was performed by ELISA using cathepsin B-specific antibodies. Tissue extracts and buffer containing cathepsin B were mixed with phosphate buffer and incubated overnight. The wells were washed with PBS and incubated with anti-cathepsin B antibody at 25°C for 3 h. The plates were washed with PBS, incubated with a second antibody, an alkaline-phosphate conjugate, and the color was developed with *p*-nitrophenyl phosphate. The concentrations of cathepsin B in these tissue extracts were determined using the standard curve for cathepsin B.

RNA extraction and Northern blotting

Frozen tissues from normal brain samples and tumors were ground to powder in liquid nitrogen and then dissolved in 4 M guanidinium isothiocyanate; total RNA was isolated as described [16]. Total tissue RNA (20 μg) from each sample was electrophoreses in formaldehyde containing 1% agarose gels and transferred to Hybond membranes (Amersham Corp., Arlington Heights, IL) by capillary action using 10× SSC buffer. The membranes were fixed by baking at 80°C for 2 h, and the blots were probed at 42°C with random-primed ^{32}P-labeled cathepsin B cDNA probes [17, 18]. The probes were labeled with α-[^{32}P]-dCTP (6000 Ci/mmol) using a random-primed labeling kit (Boehringer Mannheim Corp., Indianapolis, IN). The blots were washed at stringency conditions using 0.5× SSC in the presence of 1% SDS at 65°C, autoradiographed using Hyperfilm (Amersham Corp.), and exposed for 1–3 days at −80°C using intensifying screens. Subsequently, the blots were reprobed with a β-actin cDNA to confirm loading equalities. The results were corrected for RNA loading by densitometric normalization to the β-actin signal.

Immunohistochemistry

Cathepsin B immunoreactivity was analyzed in 10% formalin-fixed and paraffin-embedded sections by using the cathepsin B-specific polyclonal antibody (rabbit anti-human cathepsin B polyclonal antibody, Athens Research and Technology, Inc.). An appropriate concentration of the primary antibody was determined by titering the antibody using positive control tissue. Sections 4 μm thick were cut and mounted on aminoethoxysilane-coated glass slides. Cathepsin B expression was detected by using an indirect avidin–biotin–peroxidase complex method. The slides were dewaxed and blocked with normal goat serum. The secions were then incubated with rabbit anti-human cathepsin B polyclonal antibody diluted 1:300 in PBS (23.5 μg/ml) for 1 h at room temperature in a humidified chamber. After a brief wash in buffer, the tissue samples were incubated with biotinylated goat anti-rabbit second antibody and streptavidin–alkaline phosphatease (Biogenese Laboratories, San Ramon, CA). Alkaline phosphatase activity was visualized by the addition of a substrate solution consisting of naphthol AS–BI phosphate, levamisole, and fast-red TR, which forms an intense red color in the cell cytoplasm, and sections were counterstained in hematoxylin. A control study was performed by substituting a nonspecific IgG for the primary antibody.

Results

Enzyme activity assay

Cathepsin B activity was determined from extracts of normal brain and various types of brain tumor tissues at pH 5.2. Cathepsin B activity was present in normal brain and brain tumor tissue extracts (Figure 1). The activity of cathepsin B was significantly higher in

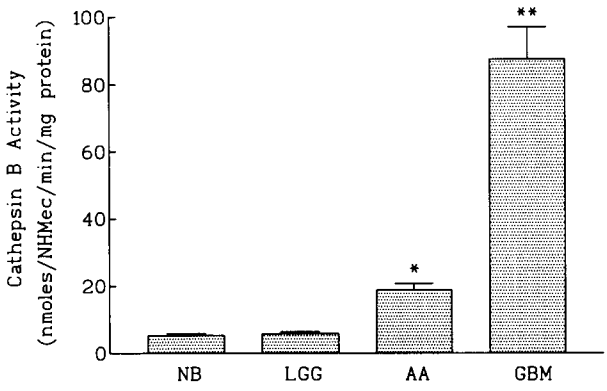

Figure 1. Activity of cathepsin B in normal brain tissue and brain tumor tissue extracts. Enzyme activity is expressed as nmoles of naphthalamide released per min per milligram of protein. Each value represents mean ± SD of five different patient samples from each group. NB, normal brain tissue; LGG, low-grade glioma; AA, anaplastic astrocytoma; and GBM, glioblasoma. * $P < 0.001$; ** $P < 0.0001$.

anaplastic astrocytoma (three-fold; $P<0.001$); and glioblastoma (10-fold; $P<0.0001$) than in normal brain tissue and low-grade gliomas. There was no significant difference in cathepsin B activity between normal brain tissue and low-grade glioma. Cathepsin B activity was completely abolished by E-64, an inhibitor of cysteine proteases, in normal brain and tumor tissue extracts (data not shown).

Western blotting

The molecular weight of cathepsin B in normal brain and tumor tissue extracts was determined by SDS–PAGE, followed by Western blotting using a specific antibody for cathepsin B (Figure 2). From the Western blot the predominant cathepsin B doublet at *M*r 25 000 and 26 000 and its precursor forms at *M*r 46 000 and 43 000 were present in normal brain and tumor tissue extracts. The *M*r 31 000 and 37 000 forms were present only in the glioblastoma tissue samples (Figure 2). The intensity of the doublet at *M*r 25 000 and 26 000 was highest in the glioblastomas and higher in the anaplastic astrocytoma than in normal brain and low-grade glioma samples. Taking into consideration the intensity of the *M*r 25 000 and 26 000 bands of all the glioma samples, it is apparent that there is an increase in cathepsin B protein with progression and histological grade of gliomas.

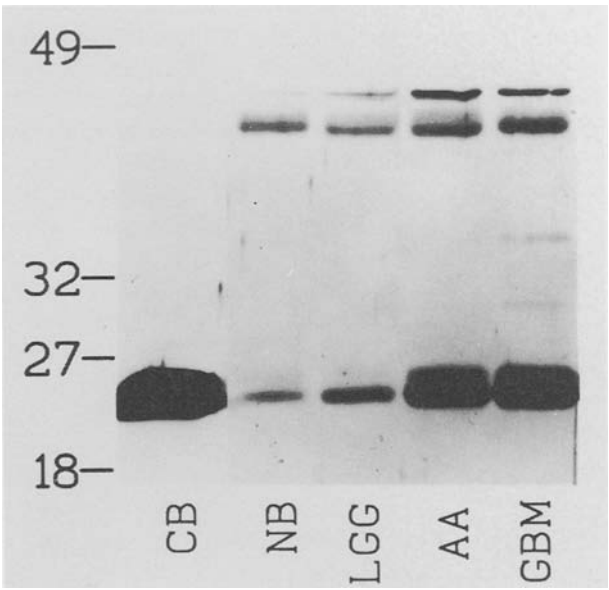

Figure 2. Western blot analysis of cathepsin B in normal brain and brain tumor tissue extracts. Protein from tissue extracts (50 μg protein) and purified cathepsin B were subjected to SDS–polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose as described in Materials and methods. CB, cathepsin B; NB, normal brain tissue; LGG, low-grade glioma; AA, anaplastic astrocytoma; and GBM, glioblastoma.

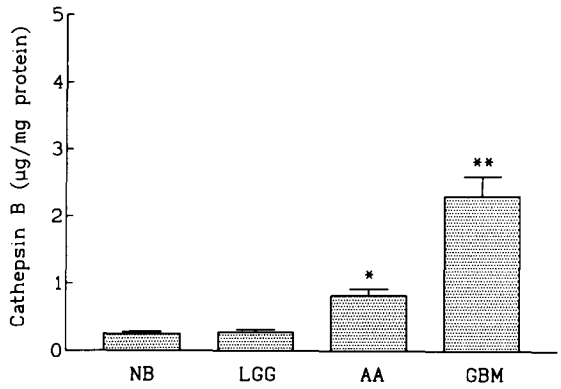

Figure 3. Cathepsin B content in normal brain and tumor tissue extracts determined by ELISA using cathepsin B specific antibodies. Data are mean values ± SD of five different samples from each group. NB, normal brain tissue; LGG, low-grade glioma; AA, anaplastic astrocytoma; and GBM, glioblastoma. * $P<0.001$; ** $P<0.0001$.

ELISA

We also quantified the levels of cathepsin B protein in normal brain tissue and tumor tissue extracts by ELISA using specific antibody for cathepsin B. Figure 3 shows that cathepsin B protein levels were higher in anaplastic astrocytoma (three-fold; $P<0.001$) and glioblastoma (nine-fold; $P<0.0001$) than in normal brain tissue and low-grade glioma samples. There was no significant difference in the amounts of cathepsin B found in normal brain tissue and low-grade glioma.

Northern blotting

Total RNA isolated from normal brain tissue and various types of brain tumor tissues were probed with labeled cathepsin B cDNA to determine the levels of cathepsin B transcripts. Northern blot analysis of the isolated cathepsin B mRNA revealed two distinct cathepsin B transcripts (4.1 and 2.2 kb) in all specimens, including normal brain tissue (Figure 4). The sizes of these transcripts are similar to those published [17, 18]. The amounts of cathepsin B mRNA were markedly higher in the glioblastoma samples and moderately higher in the anaplastic astrocytomas than in normal brain tissues and low-grade glioma tissue samples.

Further quantitation of cathepsin B mRNA was performed by laser densitometry and the values were normalized to β-actin mRNA (Table 1). We found that the levels of cathepsin B mRNA transcripts were increased 2.8-fold ($P<0.001$) in anaplastic astrocytoma, and 7.8-fold ($P<0.0001$) in glioblastoma

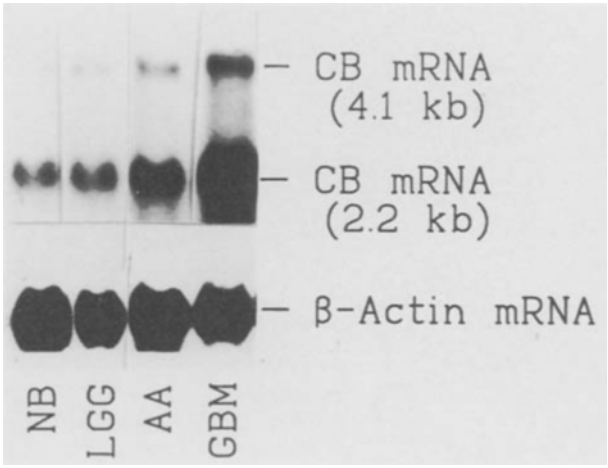

Figure 4. Northern blot analysis of cathepsin B mRNA in normal brain tissue and various brain tumor tissues. Total RNA (20 μg) was electrophoresed in a 1.5% agarose gel and transferred to nytran-modified nylon filters by capillary action. The membrane was then hybridized with a radiolabeled 3 kb cDNA probe specific for cathepsin B mRNA. After hybridization, the filter was stripped and rehybridized with a β-actin probe to check mRNA loading amounts. NB, normal brain tissue; LGG, low-grade glioma; AA, anaplastic astrocytoma; and GBM, glioblastoma.

Table 1. Relative hybridization signal for cathepsin B in human brain tumors[a]

Tissue specimens	Cathepsin B mRNA[b] (arbitary units)
Normal brain	1.0 ± 0.11
Low-grade glioma	1.1 ± 0.14
Anaplastic astrocytoma	2.8 ± 0.35*
Glioblastoma	7.8 ± 0.85**

[a] Relative hybridization signal numbers were calculated by ascribing an arbitrary value of 1 to normal brain tissue, the lowest signal seen on Northern blots for cathepsin B mRNA expression after loading equalities were normalized with β-actin. Relative hybridization signal numbers were calculated from data obtained by laser densitometry from five different patients in each group.
[b] Data are shown as mean values $\pm$ SD of five different patient samples from each group.
* $P < 0.001$; ** $P < 0.0001$.

compared with normal brain and low-grade glioma samples. There was no significant difference in the levels of cathepsin B transcripts in normal brain tissues and low-grade gliomas.

Immunohistochemical localization of cathepsin B

We determined the relative level of expression and the distribution of cathepsin B in tumor and normal brain tissue by immunohistochemical analysis using paraffin-embedded sections. Antibodies against cathepsin B showed intense immunoreactivity in tumor and endothelial cells of glioblastomas and anaplastic astrocytomas (Figure 5a and b). Low-grade astrocytoma and normal white matter astrocytes exhibited weak but detectable immunoreactivity (Figures 5c and d). No staining was seen when a nonspecific IgG was substituted for the anti-cathepsin B antibody. These results were consistent with ELISA and Northern blot analysis and demonstrated that abundant levels of cathepsin B protein and mRNA were present in glioblastoma and anaplastic astrocytoma but only low levels were found in low-grade gliomas and normal brain tissue samples.

Discussion

Although there are no previous reports on the presence of cathepsin B in human brain tumors and normal brain tissues *in vivo*, the expression of protease other than the cysteine protease superfamily, such as serine proteases (plasminogen activators) and metalloproteases (collagenases type IV) have been investigated. All of these proteases are thought to be involved in tumor invasion. Several reports have indicated differences in the production of plasminogen activators in solid brain tumors and in cell lines derived from these tumors [19–21]. The synthesis of different metalloproteases and tissue inhibitors of metalloproteases by cultured fetal astrocytes and glioma cell lines has also been reported [5, 22, 23]. For example, a metalloprotease secreted by the rat glioma cell line BT5C in serum-free medium was capable of degrading fetal rat brain aggregates [24, 25], and Caroni and Schwab [26] described a metalloprotease activity that facilitates CNS invasion in an *in vitro* model. Our recent results also demonstrated highly elevated levels of 92 kDa type IV collagenase in glioblastoma samples *in vivo* [27].

The cysteine or thiol proteases constitute a family of closely related enzymes that differ primarily in their substrate specificity and sensitivity to specific inhibitors. One of the specific substrates for cathepsin B that serves to distinguish it from the other cathepsins contains a pair of arginine residues [28]. We found that both acidic and neutral tissue extracts contained cathepsin B activity toward Nα-CBZ–Arg–Arg–4-methoxy–β-naphthalamide substrate, however the activity of the acidic extract appears to be about 20–25 times greater than that of the neutral extracts (data not shown).

Lysosomal enzymes such as cathepsin B are

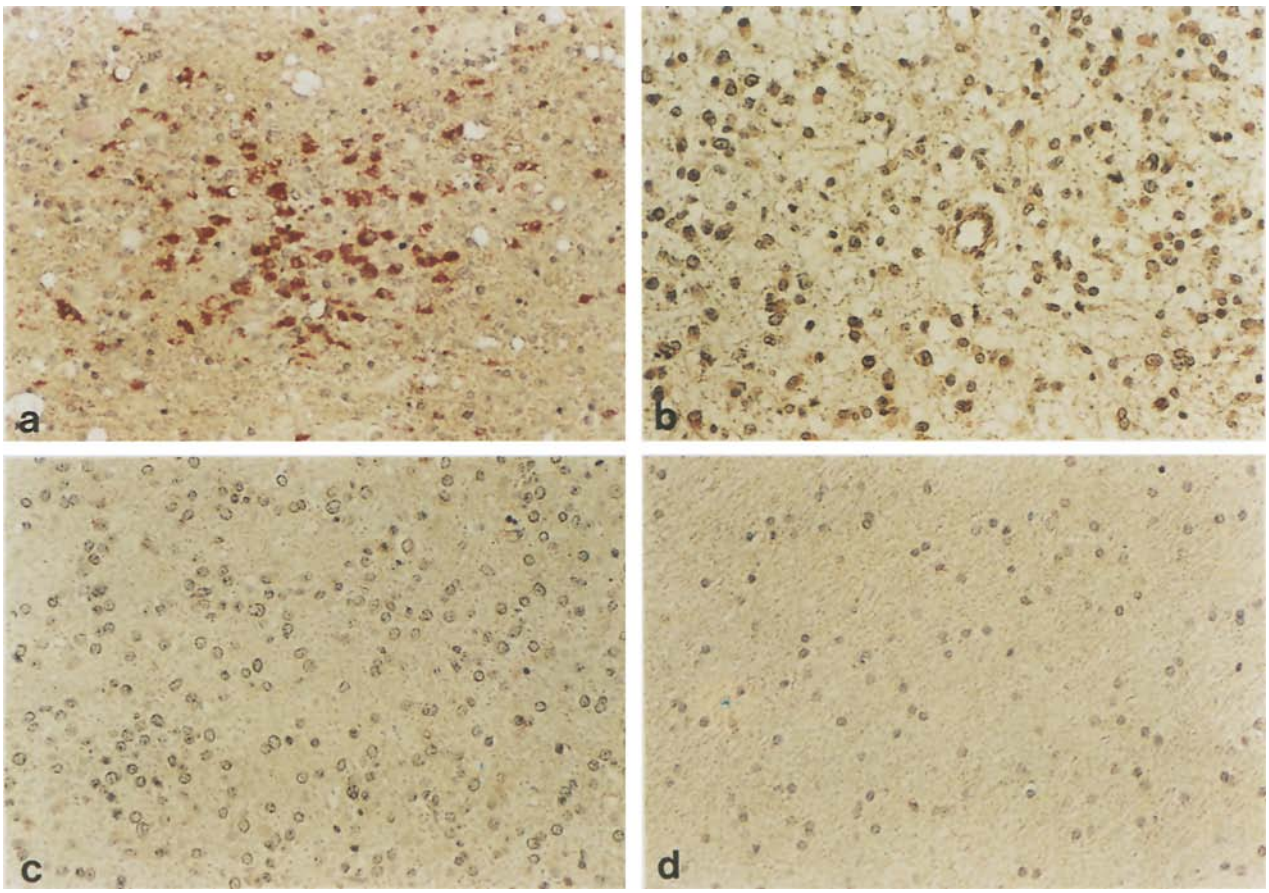

Figure 5. Immunohistolocalization of cathepsin B in various types of human astrocytomas and normal brain tissues using cathepsin B-specific antibody was performed as described in Materials and methods. a, glioblastoma; b, anaplastic astrocytoma; c, low-grade astrocytoma; d, normal brain tissue. ×260.

synthesized as inactive high-molecular-weight precursors that are proteolyticaly processed to yield active mature enzymes [29–31]. In the present study, all of the brain tumor extracts showed precursor forms of cathepsin B (*Mr* of 46 000 and 43 000). It has been reported that both procathepsin B and procathepsin L, present in the hepatic endoplastic lumen, have the same *Mr* of 39 000 and are inactive [32]. Their enzymatic activities are markedly increased after 36 h of incubation at pH 3.0. Cathepsin B and L activities were increased 60 and 210 times, respectively, at pH 3.0 due to the conversion of the proenzymes to mature enzyme forms. The increase in enzymatic activities and the conversion of the proenzymes to mature forms could be completely blocked by pepstatin, a potential inhibitor of cathepsin D. In addition, lysosomal cathepsin D can convert microsomal procathepsin B to its mature enzyme forms *in vitro* [32].

We also demonstrated that there are higher amounts of cathepsin B mRNA and protein in glioblastomas than in normal brain tissues and low-grade gliomas and that this was due to increased transcript or message synthesis reflecting an increase in the expression of the cathepsin B gene. The present study provides for the first time evidence for an association between the expression of cathepsin B protein and message and malignant progression of brain tumors. We also demonstrated that cathepsin B was localized in tumor and endothelial cells of tumor tissue. Since local invasive growth is one of the key features of primary malignant brain tumors and is accompanied by remodeling of the microvasculature and destruction of normal brain tissue [1], the invasive character of malignant astrocytomas may depend, in part, on the presence of cellular proteolytic enzyme activities for degradation of extracellular matrix components. Biochemical studies including an examination of the subcellular distribution of cathepsin B indicated the presence of enzymatically active cathepsin B in the plasma membranes of cancer cells, suggesting that cathepsin B is a membrane-associated protein in malignant cells [11, 33, 34]. This cell-associated

cathepsin B can activate proenzyme uPA and pro-type IV collagenases, resulting in degradation of the extracellular matrix [8]. Using immunohistochemical and *in situ* hybridization techniques, we demonstrated that uPA protein and mRNA are localized within astrocytoma cells and endothelial cells and are heterogeneously distributed within glioblastomas, preferentially near vascular proliferation zones and at the leading edges of tumors [21]. We also observed strong immunoreactivity of type IV collagenases (72 and 92 kDa) in tumor cells and in the vasculature of glioblastoma and anaplastic astrocytomas compared with normal brain tissue and low-grade gliomas. Thus, it appears that several ECM-degrading enzymes are overexpressed in the more malignant brain tumors and these enzymes probably play an important role in CNS invasion. The quantitative estimation of degradative enzymes such as cathepsin and their localization in human brain tumor samples might provide important new prognostic information in evaluating the degree of malignancy of brain tumors.

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