**Immunolocalization of cathepsin B in human glioma: implications for tumor invasion and angiogenesis**

**TOM MIKKELSEN, M.D., PEI-SHA YAN, M.D., KHAND-LOON HO, M.D., MANSOUREH SAMENI, M.S., BONNIE F. SLOANE, PH.D., AND MARK L. ROSENBLUM, M.D.**

Henry Ford Midwest Neuro-Oncology Center and Departments of Neurosurgery, Neurology, and Pathology, Henry Ford Hospital, Detroit, Michigan; and Department of Pharmacology, Wayne State University, Detroit, Michigan

The poor prognosis of patients with malignant gliomas is at least partially due to the invasive nature of these tumors. In this study, the authors investigated the possibility that the cysteine protease cathepsin B (CB) is a participant in the process of glial tumor cell invasion. To accomplish this, an immunohistochemical analysis was made of the localization of antibodies to CB in biopsies of five specimens of normal brain, 16 astrocytomas, 33 anaplastic astrocytomas, and 33 glioblastomas multiforme.

Staining was scored according to the percentage of positive cells and the intensity of the stain, graded from 0 to 3+. Staining for CB was not seen in any of five samples of normal brain except for occasional neuronal cell bodies and microglia. Only five (31%) of 16 astrocytomas showed a small percentage of positive cells (0.01%–3%) that were stained in a light, diffuse cytoplasmatic pattern (1+). Twenty-nine (87.8%) of 33 anaplastic astrocytomas showed positive light, granular staining in 2% to 40% of cells. In anaplastic astrocytoma, the staining within a tumor was heterogeneous with intensities of 1+ (17%), 1+ to 2+ (29%), or 2+ (55%). In contrast, all 33 (100%) glioblastomas were positive in 10% to 90% of cells. The staining was present in a coarse, granular pattern with an intensity of 2+ (12%) or 3+ (88%). Tumor cells infiltrating into brain adjacent to malignant gliomas stained positively in 26 cases that could be evaluated for glioblastoma multiforme; these invading cells frequently followed penetrating blood vessels as typical “secondary structures of Scherer.” Moderate to intense CB staining associated with endothelial proliferation in high-grade tumors was also observed, especially in regions of tumor infiltration into adjacent normal brain. These results provide evidence consistent with the hypothesis that CB is functionally significant in the process of tumor invasion and angiogenesis in the clinical progression of the malignant phenotype in astrocytomas.

**KEY WORDS**  • cathepsin B  • glioma  • angiogenesis  • invasion
MMP-2 and the cysteine protease CB are both increased. In human colorectal adenocarcinoma high levels of expression of both CB and urokinase define a morphologically and clinically aggressive subset of cases. Such observations suggest the possibility of functional interactions among the proteases CB, MMP-2, and urokinase. It should be noted that elevated CB expression has also been described in earlier stage colorectal tumors.

Because the expression of metalloproteases and urokinase has been reported to correlate with the progression of human brain tumors, the objectives of the present study were to analyze the expression of CB in a series of astrocytomas, anaplastic astrocytomas, and glioblastomas. A single report has described the secretion of CB by gliomas. The expression of CB has also been described in earlier stage colorectal tumors.

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**Materials and Methods**

**Tumor Classification**

Individual tumors were classified as pilocytic astrocytoma, anaplastic astrocytoma, or glioblastoma multiforme based on the new World Health Organization (WHO) classification of brain tumors.

**Tissue Specimens**

Five normal brains, which included cortex and white matter, were obtained from autopsy cases without any evidence of brain tumor or other brain disease. Brain tumors were obtained from supratentorial surgical resection specimens and included 15 fibrillary astrocytomas, one pilocytic astrocytoma, 33 anaplastic astrocytomas, and 33 glioblastomas multiforme. Twenty-six brain tissue specimens adjacent to the tumor core consisting of normal-appearing cortex with scattered tumor cells were also included and are designated as infiltrative areas in this study (Fig. 1). All tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut into 6-μm sections.

**Antibody Preparation**

For the preparation of monospecific anti-CB, immunoglobulin (Ig)G antisera were raised in New Zealand White male rabbits against a CB-derived synthetic peptide (residues 135–147) as we described previously. This peptide is unique to CB, as determined by computer analysis against DNA sequences in the Genbank, and resides in a surface loop of CB. An IgG fraction was purified and stored at –20°C. Details on the characterization and specificity of the anti-CB peptide IgG were described previously. The anti-CB IgG used for these studies recognizes both inactive proforms of CB and the active mature forms (single chain and double chain) of CB.

**Histological Analysis**

To compare the results of immunohistochemical study with malignancy features and to validate the grade classification of cases...
selected, five standard morphological features of malignancy were assessed on hematoxylin and eosin–stained sections: hypercellularity, nuclear pleomorphism, mitoses, vascular endothelial proliferation, and necrosis, as included in the recently amended WHO criteria for the diagnosis of primary tumors of the central nervous system.1

Slide Scores

Slides were scored and a blinded review was undertaken by a neuropathologist. Staining intensity, graded as negative (−), weak (+), moderate (++), or strong (+++) and the number of tumor cells that were positively stained, was expressed as a percentage of the total number of tumor cells estimated from 10 to 15 representative fields from each section. Mitoses were graded as none (0), 1 to 2 per hpf (+), 3 to 5 per hpf (++), or greater than 5 per hpf (+++) after counting 10 representative fields and averaging the number of mitoses seen.

Immunohistochemical Analysis

The immunohistochemical avidin–biotin–peroxidase complex technique was used as described in a previous paper.4 After routine deparaffinization and rehydration, tissue sections were incubated for 10 minutes with 3% hydrogen peroxide in distilled water to inactivate endogenous peroxidases. Slides to be stained were pretreated in a microwave oven to allow access of fixed-embedded tissue antigen by these two antibodies.10 The slides with deparaffinized tissue sections were placed in a glass jar filled with 10 mM sodium citrate buffer (pH 6.0) and boiled for 3 to 5 minutes in a microwave oven with a capacity of 650 to 720 W. Boiling was repeated another 5 minutes after refilling with buffer. Slides were cooled at room temperature for 20 minutes and then rinsed in phosphate-buffered saline (PBS).

For anti–human macrophage antibody KP1 (CD68), tissue sections were digested by 0.25% trypsin in 0.25% calcium chloride buffer at 37˚C for 30 minutes followed by rinsing with PBS. Sections were then incubated with 10% normal goat or horse serum at room temperature for 30 minutes to block nonspecific binding. The slides were incubated with primary antibodies as follows: anti-CB (1:500 dilution in PBS, overnight at room temperature), anti-Ki-67 (MIB-1) (1:50 dilution in PBS, overnight at 4˚C), and anti-macrophase (KP1) (1:200 dilution in PBS, overnight at 4˚C). After three washes in PBS, the slides were incubated for 30 minutes at room temperature with biotinylated secondary antibodies (1:200 dilution in PBS), washed, and incubated for 45 minutes at room temperature with the Vectastain AB Kit (Vector Laboratories, Burlingame, CA). Finally, the sections were washed, reacted with diaminobenzidine in 0.1 M Tris buffer (pH 7.6) with 0.03% hydrogen peroxide, rinsed in tap water, counterstained, and mounted.

Results

Immunohistochemical Analysis Using Staining for Cathepsin B

The histological features of astrocytomas, anaplastic astrocytomas, and glioblastomas are summarized in Table 1. In five normal brain tissues from autopsy, CB staining was present in normal microglia. These were identified as displaying slender, elongated nuclei and ramified cytoplasmic processes. This CB staining was also seen in neurons but not in astrocytes or oligodendroglial cells (Fig. 2A).

Astrocytoma. Eleven (68.8%) of 16 astrocytomas did not express CB. The remaining five cases had a few cells (range 0%–3%) that were stained in a light, diffuse non–granular cytoplasmic pattern (+) (Fig. 2B).

Anaplastic Astrocytoma. Compared to astrocytoma, anaplastic astrocytoma showed increased expression of CB, both in intensity and number of cells stained. The proportion of positive cells varied from 2% to 20%. The positive staining in anaplastic astrocytoma was finely granular and cytoplasmic, (+) in five cases, (++) in 24, with only four cases unstained (−) (Fig. 2C).

Glioblastoma. Twenty-nine (87.8%) of 33 glioblastomas multiforme showed strong expression of CB (+++) (Fig. 1A and B). Despite extensive CB staining (Fig. 1C), these cells did not stain with the macrophage marker KP1 (Fig. 1D).

Vascular Elements. Vascular changes were not seen in astrocytoma. Endothelial proliferation in glioblastomas was more abundant than that seen in other grades. In glioblastoma the vascular lumina were surrounded by two or more layers of haphazardly arranged or piled-up endothelial cells that often showed cytological atypia. Some glioblastomas demonstrated vascular glomeruli (Fig. 3A). It was interesting to note that 24 (72.7%) of 33 glioblastomas multiforme and eight (24%) of 33 anaplastic astrocytomas showed positive staining for CB in blood vessels that demonstrated endothelial cell proliferation (Fig. 3B). Although the intensity of staining in these proliferative

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**TABLE 1**

*Histomorphological features of astrocytic tumors in specimens of human brain*

<table>
<thead>
<tr>
<th>Feature</th>
<th>Degree of Abnormality</th>
<th>Astrocytoma</th>
<th>Anaplastic Astrocytoma</th>
<th>Glioblastoma Multiforme</th>
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<tr>
<td>cell density</td>
<td>+</td>
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<td>6</td>
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<td></td>
<td>++</td>
<td>4</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>pleomorphism</td>
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<td>16</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>0</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>mitosis</td>
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<td>0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>17</td>
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<td>12</td>
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<td></td>
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<td>5</td>
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<td>0</td>
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* Staining intensities are defined as: negative, −; weak, +; moderate, ++; and strong, +++.
cells was less than that in tumor cells, most of them displayed diffuse staining throughout the cytoplasm.

**Immunological Analysis Using Staining for Monoclonal Antibody MIB-1**

Staining with monoclonal antibody MIB-1 against Ki-67 nuclear antigen was compared with the results of CB staining.

**Astrocytoma.** Astrocytomas showed a very low MIB-1 labeling index. In seven cases no labeling was seen, and in the remainder MIB-1 indices ranged from 0% to 3% (mean 1.8%; standard deviation (SD) 1.0).

**Anaplastic Astrocytoma.** Anaplastic astrocytomas showed an intermediate labeling index ranging from 2% to 35% (mean 11.2%; SD 8.4).

**Glioblastoma.** The glioblastomas multiforme expressed a high MIB-1 index, which ranged from 12% to 70% (mean 36.9%; SD 16.5).

**Vascular Elements.** Positive staining with MIB-1 was also present in proliferative endothelial cells in eight (24%) of 33 anaplastic astrocytomas and 24 (73%) of 33 glioblastomas multiforme. None of the astrocytomas showed endothelial proliferation. Macrophages and mononuclear cells did not stain with MIB-1, and no positive MIB-1 staining was found in either normal cortex or white matter from five autopsy cases. There appeared to be a correlation between the staining of CB and MIB-1 in intensity and localization. In general, tumors with a high MIB-1 labeling index had a high percentage of tumor cells with positive CB staining and, in most cases, it appeared that the CB-positive cells were those stained with MIB-1. Cytoplasmic staining of CB in some proliferative endothelial cells was also accompanied by MIB-1 labeling.

**Immunological Analysis Using Staining With Monoclonal Antibody KP1**

Staining with monoclonal antibody KP1, formed against a lysosomal fraction of human lung macrophages, was used to differentiate the macrophages or mononuclear cells from tumor cells staining with CB. In normal brain from autopsy, microglia, thought to be a local macrophage cell type that displayed slender, elongated nuclei and ramified cytoplasmic processes, were also stained with KP1 (data not shown). In all three types of glial tumors examined, some macrophages or mononuclear cells (KP1 positive) near vessels also stained with CB (Fig. 1D). Approximately one-half of the cases of anaplastic astrocytoma and glioblastoma multiforme had scattered KP1-positive cells within the tumor, but only four cases of astrocytoma had KP1 staining. The KP1-positive cells were more often limited to the perivascular space or were adjacent to necrotic areas. Those cells that were morphologically defined as round cells with round nuclei often showed black granular staining; they were not labeled by MIB-1. Astrocytic tumor cells and proliferative endothelial cells did not react with this antibody, and KP1-positive cells were not seen in the infiltrating areas in peritumoral normal brain where CB-positive cells were frequently seen (Fig. 1B and D).
Discussion

The process of glial tumor invasion has been studied in vitro for some time, but the molecular pathophysiology has not been examined. It is presumed that the processes of disordered adhesion, motility, and proteolysis are involved in glial tumor invasion. Extracellular matrix alterations have been described in model systems of glioma invasion as have motility factors in glioma cell lines. Several protease classes have been depicted in primary glial tumor extracts and cell lines, including matrix metalloproteases and plasminogen activator. These studies do not describe the tumor localization of proteases. The present study on CB is the first to report the regional localization of a protease, including its presence at the infiltrating margin of the primary tumor. Although lower-grade gliomas tend to be diffuse and locally infiltrative, the degree and destructive nature of the invasion of malignant gliomas is in contrast to that of the lower grade lesions. These observations suggest that CB could play a functional role in the invasive process.

Staining of human tumor specimens with monospecific antibodies to CB or with probes for CB messenger RNA has provided further evidence that CB might participate in tumor invasion. Staining for CB is most intense in cells at the leading invasive edges of bladder and prostate tumors, respectively. An inverse correlation between CB staining and laminin staining in bladder tumors might implicate CB in the degradation of this basement membrane protein. Laminin and fibronectin have been described in peritumoral brain derived from normal cells. Cathepsin B has been shown to degrade purified laminin, fibronectin, and type IV collagen. In colon carcinoma, high levels of staining for CB correlate inversely with patient survival, suggesting a functional role for CB in colon carcinoma progression.

Conclusions

Our results clearly demonstrate that CB is expressed in glial tumor cells, with expression related to the degree of malignancy of these cells, and that CB is seen in the infiltrating tumor cells and proliferative vascular endothelium of the infiltrating margin. This distribution of CB staining cannot be accounted for by contaminating infiltration by macrophages. Finally, the localization of CB-positive endothelial cells in the tumor and near the infiltration zone implicates CB in the process of tumor angiogenesis, which may itself have relevance for tumor infiltration.

The CB antibody used in the present studies recognizes an antigenic epitope that is present in all forms of the enzyme, including the proform, a latent or functionally inactive form of the enzyme. We have presented data indicating that CB immunohistochemical expression is associated with malignant progression of human gliomas. To characterize the regulation of expression and to determine the mechanism of activation of this enzyme, we are engaged in studies of expression by Northern and Western blot tests as well as subcellular localization and activity.
studies. Cathepsin B staining with the antibody that rec-
ognizes both active and latent enzyme forms will be cor-
related with activity studies and immunostaining studies
with antibodies specific for the proform of the enzyme.

In further studies we observed increases in expression of CB (messenger RNA, protein, and activity) as well as
alterations in the subcellular localization of CB in glioma
specimens.21 Several matched normal brain–tumor pairs
were examined and a slightly greater than 10- to 14-fold in-
crease in CB activity was noted (J Rozhin and BF
Sloane, unpublished data). Further characterization of the
mechanism of regulation of CB in tumors and the func-
tional significance of this enzyme in invasion models
will require additional confirmation; these studies are un-
der way. We suggest that CB should be considered a target
for development of an antiinvasive brain tumor treatment
and that CB might be used for immunohistological detection
of the invasion of astrocytoma cells.

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Address reprint requests to: Tom Mikkelson, M.D., c/o Editorial
Office, Department of Neurosurgery, Henry Ford Hospital, 2799
West Grand Boulevard, Detroit, Michigan 48202.