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Cathepsin B Expression and Localization in Glioma Progression and Invasion

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Abstract

The poor prognosis of human malignant gliomas is due to their invasion and recurrence, the molecular mechanisms of which remain poorly characterized. We have accumulated substantial evidence implicating the cysteine protease cathepsin B in human glioma malignancy. Increases in cathepsin B expression were observed throughout progression. In primary brain tumor tissue, transcript abundance (Northern blot analysis) increased in low-grade astrocytoma to high-grade glioblastoma from 3- to 6-fold, respectively, above normal brain levels. This increase correlated with increases in protein abundance (from + to ++++) as measured by immunohistochemistry. Furthermore, in glioblastoma cell lines increases in transcript abundance (ranging from 3- to 12-fold) were accompanied by increases in enzyme activity (44-133 nmol/min x mg protein). Altered subcellular localization was observed both immunohistochemically and by indirect immunofluorescence confocal microscopy and was found to correlate with increased grade. In addition, this increase in cathepsin B expression and altered subcellular localization correlated with histomorphological invasion and clinical evidence of invasion as detected by magnetic resonance imaging. These data support the hypothesis that cathepsin B plays a role in human glioma progression and invasion.

Introduction

An association between cathepsin B and malignancy has been reported for human colon (1–5), breast (6–8), prostate (9, 10), and bladder (11) tumors. Changes in expression as measured by changes in mRNA, protein, and activity levels (1–11) as well as alterations in activation and processing (12, 13) and intracellular trafficking (8) have been reported. Recent studies on human bladder (11), prostate (9, 10), and colon carcinoma (5) have demonstrated a heterogeneity in staining for cathepsin B protein, mRNA, and in cathepsin B activity, with the most intense staining and highest activity at the invasive, leading edge of the tumors. These data suggest that the regulation of cathepsin B in tumors may be altered at more than one level and that these alterations may contribute to the invasive phenotype. Prompted by these observations we have examined the expression and localization of cathepsin B in glioma and related the results to the degree of tumor progression. Our data demonstrate changes in cathepsin B expression and subcellular localization that correlate with increased grade, local invasion, and clinical evidence of invasion thereby implicating cathepsin B in both glioma progression and invasion.

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Materials and Methods

Tissue Samples. Immediately after surgical removal, specimens were taken for pathological diagnosis and fixation and embedding while the remaining tissue was snap frozen in liquid nitrogen. Glioma specimens were categorized by grade (I, II, III, and IV) according to the WHO grading system (14). Informed consent was obtained from all patients or the guardian of that patient.

Northern Blots. To determine whether increases in cathepsin B transcript abundance correlated with malignant progression in gliomas, Northern blot analyses were performed using total RNA from normal brain, astrocytoma and oligodendroglioma (grade II), anaplastic astrocytoma (grade III), and glioblastoma multiforme (grade IV) biopsy samples and glioblastoma cell lines. RNA was extracted using the guanidium thiocyanate procedure (15), quantitated by measuring absorbance at 260 nm, electrophoresed through a 1.2% formaldehyde agarose gel in the presence of ethidium bromide, and transferred to Hybond N (16). Following prehybridization, the filter was hybridized to a 32P-radiolabeled cathepsin B probe (2), stringency washed, and exposed to X-ray film. The abundance of the 2.1-kilobase cathepsin B was determined by quantifying hybridization signals on X-ray film using densitometric analysis as reported previously (16). The abundance for each sample was corrected for variations in sample loading by normalizing the values to the 18S RNA signal. The percentage transcript abundance for each sample was expressed as a percentage of the maximum integral value (U87 = 100%). The fold increase in abundance was calculated as a fold increase above levels seen in normal tissue. The mean value is presented for the tumors.

Immunohistochemistry. Antisera were raised in rabbits (New Zealand White males) against a cathepsin B-derived synthetic peptide (residues 135–147) as described previously (17). IgG fractions were purified and stored at −20°C. The specificities of the IgGs for cathepsin B have been confirmed by slot blotting and immunoblotting using purified human liver and tumor cathepsin B as well as homogenates and/or extracts of human normal and tumor tissues (17). The monospecific anti-cathepsin B IgGs recognize procathepsin B and single- and double-chain forms of the mature enzyme in immunoblots (17). Immunohistochemical analyses (18, 19) were performed. Paraffin-embedded tissue sections were subjected to routine deparaffinization and rehydration. Sections were incubated for 10 min with 3% hydrogen peroxide in distilled water to inactivate endogenous peroxidases. Slides were then placed in a Coplin jar with 10 mM sodium citrate buffer (pH 6.0) and boiled for 3–5 min in a microwave oven with a capacity of 650–720 W. After the buffer was changed, boiling was repeated for an additional 5 min. Slides were permitted to cool to room temperature (20 min) followed by rinsing in PBS. All subsequent steps were performed at room temperature. Sections were then incubated with 10% normal goat or horse serum for 30 min to block nonspecific binding sites. The slides were incubated overnight with a 1:50 dilution of primary anti-cathepsin B antibody in PBS. After three washes in PBS buffer the slides were incubated for 30 min with biotinylated secondary antibody (1:200 in PBS) and then washed and incubated for 45 min at room temperature with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Finally, the sections were washed and reacted with diaminobenzidine in 0.1 M Tris buffer (pH 7.6) with 0.03% hydrogen peroxide, followed by rinsing in tap water, counterstaining, and mounting. Slides were scored and blindly reviewed by a neuropathologist. Staining intensity was graded as negative (−), weak (+), moderate (++), or strong (+++).

Enzyme Activity Assays. The activity of cathepsin B (Vmax) in cell lines and tissues was determined against Z-Arg–i-Arg–NH2Me as we have described (20). Protein and DNA contents were determined using the bicinchoninic acid method of Fierce Chemical Co. (Rockford, IL) with bovine serum albumin as a standard.

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Confocal Microscopy. Intracellular cathepsin B was localized using a previously described modification (8) of the general immunocytochemical methodologies described by Willingham (22). Antisera were raised in rabbits (New Zealand White males) against the mature double-chain form of human liver cathepsin B (17). The specificities of the IgGs were the same as the cathepsin B antipeptide IgGs. Cells grown on glass coverslips were fixed with methanol (−20°C) for 5 min. After being washed with PBS, cells were blocked with 2 mg/ml bovine serum albumin in PBS. All subsequent antibody and wash solutions contained 0.1% saponin. Cells were incubated with primary antibodies (rabbit anti-human liver cathepsin B IgG plus, in the double-labeling studies, mouse anti-β-tubulin IgG1) for 2 h and washed. In controls, premun serum was substituted for the primary antibody. After blocking with normal donkey serum (5% in PBS-0.1% saponin) cells were incubated for 1 h with fluorescein- or Texas red-conjugated affinity-purified donkey anti-rabbit IgG and fluorescein- or Texas red-conjugated affinity-purified donkey anti-mouse IgG at 20 μg/ml. Cells were then washed, mounted with Slow Fade antifade reagent, and observed on a Zeiss LSM 310 microscope.

MRI4 Scans. Scans were blindly reviewed by a neurologist and classified as either focal with no evidence of regional invasion, locally invasive, or diffuse with widespread invasion. In addition, each scan was determined as to whether the tumor was enhancing or nonenhancing with respect to gadolinium contrast agent.

Results and Discussion

Northern blot hybridization with a 32P-radiolabeled cathepsin B probe revealed a major 2.1-kilobase transcript and a minor 4.0-kilobase transcript (Fig. 1), as has been observed previously in other normal and tumor brain tissues (2, 20). Normalized densitometric analysis was performed using 18S rRNA levels (16) as the standardizing internal control. The percentage of transcript abundance was normalized to U87 RNA levels as 100%. The abundance of the 2.1-kilobase cathepsin B transcripts in glioblastomas was approximately twice that seen in a low-grade glioma and anaplastic astrocytomas which in turn had increases in transcript abundance relative to the normal brain specimen (Table 1). The fold increases in individual tumors were consistent with the diagnosis of a glioblastoma with the exception of frank necrosis. This sample therefore has been reclassified to represent at least an anaplastic astrocytoma and was included in this grade for data analysis. Thus the sample used for the initial histomorphological examination was not representative of the most malignant part of the tumor, a sample of which was analyzed in our Northern blot. These results emphasize the problem of sample heterogeneity within glioma tumors. Importantly, our data demonstrate the potential usefulness of cathepsin B as a diagnostic marker.

Immunohistochemical analysis (19) was performed using a polyclonal anti-cathepsin B antibody which detects both mature and precursor forms of cathepsin B (8, 17). To ensure that cathepsin B staining occurred in tumor cells and not in infiltrating macrophages, adjacent sections for all samples were stained with a macrophase-specific monoclonal antibody KP-1 (data not shown). Sections of tumors of various grades were stained and compared with the expression pattern observed in normal brain. Staining of sections of normal brain showed significant expression of cathepsin B in neuronal cell bodies and processes. However, no positive staining of astrocytes or oligodendrocytes was observed (Fig. 2A). Scattered positive staining was noted in microglia in both white and gray matter. Positive cathepsin B staining in low-grade glioma was found to be weak (+), granular, and perinuclear (Fig. 2B). Cathepsin B expression was intermediate (+ +) in anaplastic astrocytomas (Fig. 2C). In glioblastomas it was strong (+ + +), and the number of cells staining positive for cathepsin B ranged from 10 to 90% in different areas within the
CATHEPSIN B IN GLIAL TUMORIGENESIS

Table 1 Human gliomas and cell lines: cathepsin B transcript abundance and enzyme activity

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fold increase (2.1-kilobase transcript)</th>
<th>Cell lines</th>
<th>Fold increase (2.1-kilobase transcript)</th>
<th>Activity (nmol/min X mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>U251MGn</td>
<td>3</td>
<td>44</td>
</tr>
<tr>
<td>Low-grade glioma</td>
<td>2 ± 2</td>
<td>U251Mgp</td>
<td>11</td>
<td>95</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td></td>
<td>U87</td>
<td>12</td>
<td>133</td>
</tr>
<tr>
<td>Glioblastoma multiforme</td>
<td>6 ± 4</td>
<td>HF66</td>
<td>ND*</td>
<td>351a</td>
</tr>
</tbody>
</table>

* ND, not determined.

A 14-fold increase in cathepsin B activity was observed in tumor vs accompanying normal brain sample of patient HF66 (data not shown).

The fold increase in transcript abundance for the samples presented in Fig. 1 is presented as the mean ± SD for n = 1 for low grade glioma, n = 9 for anaplastic astrocytoma, and n = 6 for glioblastoma multiforme, where n is the number of specimens.

tumors (Fig. 2, D and E). The intensity of staining in these cells was greatly increased (Fig. 2, D and E). We have now performed immunohistochemical analyses on a large number of tumors (16 low-grade tumors, 33 anaplastic tumors, and 33 glioblastomas). These analyses indicate that the results reported here are representative of cathepsin B expression for the various grades of glioma. Increased levels of cathepsin B protein accompany increases in transcript abundance, and both increase during glioma progression.

Whereas most glioblastomas have staining patterns as indicated (Fig. 2, D and E), several tumors also displayed a pattern in which the occasional capillary was surrounded by either a single layer of tumor cells that expressed cathepsin B (as in Fig. 2F) or endothelial cells in tumor vessels that expressed cathepsin B (data not shown). Our observations suggest that cathepsin B expression in these tumor and endothelial cells may play a role in angiogenesis in glioblastomas (23).

If cathepsin B plays a role in glioma invasion, one would expect high expression at the invading edge of the tumor. In a high-power magnification (Fig. 2H) of the interface between normal gray matter and tumor (Fig. 2G), positively staining cells were observed in the gray matter. Therefore, expression in the peripheral invading edge of the tumor places cathepsin B at the site of local invasion. Furthermore, cathepsin B-expressing tumor cells surrounded vessels in areas of infiltrated brain (Fig. 2F) which represent the histological invasion pattern known as secondary structures of Scherer (24).

To determine whether increases in the enzyme detected correlated immunohistochemically with increased function (since the antibodies detect both pro and mature forms), we assayed cathepsin B activity (20) in frozen sections of normal and tumor tissues and glioblastoma cell lines (Table 1). Cathepsin B activity was found to be increased 14-fold in glioblastoma versus normal for the matched normal/tumor pair HF66 (Table 1 legend) consistent with an 11-fold increase in transcript abundance and elevated protein levels (Fig. 2, A and D). Enzyme activity was also elevated 8-fold in glioblastoma versus normal for an additional matched pair HF140 (data not shown). Increasing enzyme activity in the cell lines correlated with increasing transcript abundance (Table 1).

Because the immunohistochemical staining suggested alterations in cellular distribution of cathepsin B in glioblastomas compared with lower grade tumors and because alterations in the intracellular trafficking of cathepsin B have been implicated in tumorigenesis of other tumor types (8), we have delineated the subcellular distribution of cathepsin B using immunofluorescent staining (8) and confocal microscopy (Zeiss LSM 310) analysis. Sections were double stained for tubulin and cathepsin B using secondary antibodies conjugated to either Texas red or fluorescein. Controls were incubated with preimmune serum rather than primary antibody. In U251MGn cells, the staining for cathepsin B was localized in the perinuclear region (Fig. 3A). In contrast, in those tumor cells that have higher cathepsin B activity, staining for cathepsin B was distributed throughout the cells in both cytoplasmic processes and perinuclear regions (Fig. 3, B–D). Only a weak background fluorescence was observed in the presence of preimmune IgG and secondary antibodies (data not shown). Cathepsin B is normally localized in lysosomes in the perinuclear regions of cells (8) as observed in the U251MGn cells. However, a redistribution of lysosomes toward the cell periphery


analysis of gadolinium-enhanced T1- and T2-weighted MRI scans is used to indicate regions of tumor growth and infiltration. Low-grade and anaplastic astrocytomas most often present as focal and nonenhancing lesions, whereas malignant glioblastomas present as local and/or diffuse and enhancing lesions (Fig. 4). Often, however, MRI scans are difficult to interpret following the use of radiation therapy which, along with chemotherapy, is the standard method of postsurgical treatment. Because MRI scans cannot be relied upon solely, this necessitates the use of other diagnostic indicators. We have correlated the staining intensity and transcript abundance of cathepsin B with clinical features of invasion as determined by MRI (Table 2). Elevated cathepsin B transcript and protein abundance correlated with local or diffuse and enhancing images which in turn correlated with increased grade of tumor, indicating that enhanced cathepsin B expression correlated with clinical evidence of invasion. These data suggest that enhanced levels of cathepsin B might be predictive of invasion in vivo.

Table 2 Correlation of cathepsin B transcript abundance, staining intensity, and MRI invasion patterns

<table>
<thead>
<tr>
<th>Grade</th>
<th>Sample</th>
<th>Transcript abundance (%)</th>
<th>Staining intensity</th>
<th>Clinical invasion by MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>HF34</td>
<td>7.3</td>
<td>+</td>
<td>Focal and nonenhancing</td>
</tr>
<tr>
<td>AA</td>
<td>HF26</td>
<td>7.6</td>
<td>–</td>
<td>Focal and nonenhancing</td>
</tr>
<tr>
<td>AA</td>
<td>HF17</td>
<td>16.0</td>
<td>–</td>
<td>Focal and nonenhancing</td>
</tr>
<tr>
<td>AA</td>
<td>HF47</td>
<td>17.3</td>
<td>+ /++</td>
<td>Focal and nonenhancing</td>
</tr>
<tr>
<td>AA</td>
<td>HF22</td>
<td>18.9</td>
<td>–</td>
<td>Focal and enhancing</td>
</tr>
<tr>
<td>AA</td>
<td>HF1</td>
<td>53.2</td>
<td>ND^a</td>
<td>Diffuse and enhancing</td>
</tr>
<tr>
<td>GBM</td>
<td>HF76</td>
<td>31.5</td>
<td>+++</td>
<td>Diffuse and enhancing</td>
</tr>
<tr>
<td>GBM</td>
<td>HF31</td>
<td>33.5</td>
<td>+++</td>
<td>Local and enhancing</td>
</tr>
<tr>
<td>GBM</td>
<td>HF3</td>
<td>51.7</td>
<td>+++</td>
<td>Diffuse and enhancing</td>
</tr>
<tr>
<td>GBM</td>
<td>HF50</td>
<td>85.9</td>
<td>+++</td>
<td>Diffuse and enhancing</td>
</tr>
<tr>
<td>GBM</td>
<td>HF66</td>
<td>90.0</td>
<td>+++</td>
<td>Diffuse and enhancing</td>
</tr>
</tbody>
</table>

^a ND, not done.

Fig. 3. Subcellular localization of cathepsin B. Immunocytochemical colocalization of intracellular cathepsin B and β-tubulin in U251MGn (A), U251MGp (B), U87 (C), and HF66 (D) glioblastoma cell lines. Localization of cathepsin B was perinuclear in the less invasive cell line U251MGn, whereas cathepsin B staining was present throughout the cytoplasm, including the cell processes in the more invasive cell lines U251MGp, U87, and HF66. Tubulin staining is green in B and C and red in A and D; cathepsin B staining is red in B and C and green in A and D. The staining was repeated 3 times with comparable results. × 1000.

Fig. 4. MRI scans. Patterns of glioma invasion as assessed by gadolinium-enhanced T1- and T2-weighted MRI scans. A, normal brain; B, patient HF26, focal and nonenhancing astrocytoma; C, patient HF33, local and enhancing glioblastoma; D, patient HF66, diffuse (arrow) and enhancing glioblastoma.
Several lines of evidence (Northern blot analysis, immunohistochemical staining, and activity assays) presented in this report support the hypothesis that cathepsin B is a novel marker in glioma progression. Furthermore, alterations in subcellular localization and the expression of cathepsin B at the invading tumor edge are suggestive of a role for this enzyme in local glioma invasion. In conjunction with MRI scans and traditional histomorphological grading, cathepsin B may serve as an important diagnostic marker for human gliomas. Furthermore, our preliminary studies with a series of cathepsin B inhibitors show significant inhibition of this enzyme in vitro and suggest that this enzyme may eventually prove to be an important therapeutic target in glioma patients.

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References

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