The Clinical Significance of Cathepsin S Expression in Human Astrocytomas

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Early local invasion by astrocytoma cells results in tumor recurrence even after apparent total surgical resection, leading to the poor prognosis associated with malignant astrocytomas. Proteolytic enzymes have been implicated in facilitating tumor cell invasion and the current study was designed to characterize the expression of the cysteine proteinase cathepsin S (CatS) in astrocytomas and examine its potential role in invasion. Immunohistochemical analysis of biopsies demonstrated that CatS was expressed in astrocytoma cells but absent from normal astrocytes, oligodendrocytes, neurones and endothelial cells, microglial cells and macrophages were also positive. Assays of specific activity in 59 astrocytoma biopsies confirmed CatS expression and in addition demonstrated that the highest levels of activity were expressed in grade IV tumors. CatS activity was also present in astrocytoma cells in vitro and the extracellular levels of activity were highest in cultures derived from grade IV tumors. In vitro invasion assays were carried out using the U251MG cell line and the invasion rate was reduced by up to 61% in the presence of the selective CatS inhibitor 4-Morpholineurea-Leu-HomoPhe-vinylsulphone. We conclude that CatS expression is up-regulated in astrocytoma cells and provide evidence for a potential role for CatS in invasion. (Am J Pathol 2003, 163:175–182)

Astrocytomas are the commonest primary brain tumors and within this group the malignant grade III and IV tumors predominate (anaplastic astrocytomas and glioblastomas). Despite their non-metastatic nature, prognosis is poor because tumor infiltration of surrounding brain leads to recurrence even after apparently radical surgery. Multiple stereotactic biopsies of astrocytomas have demonstrated isolated tumor cells at considerable distances from the main tumor mass,¹ and recent investigations² have shown that tumor cells may be cultured from histologically normal brain at a distance greater than 4 cm from the gross tumor. Astrocytoma invasion is an active process that involves interactions with the host extracellular matrix (ECM), proteolytic modification of the ECM, and migration of the tumor cells into the modified matrix.³ Members of the metalloproteinase, serine proteinase, and cysteine proteinase superfamilies have been linked to glioma invasion.⁴

The cysteine proteinase cathepsin B (CatB) is expressed in gliomas in vitro⁵ and immunohistochemical studies have demonstrated its presence in astrocytomas and glioblastomas in contrast to its absence from astrocytes in normal brain.⁶,⁷ The expression and activity of CatB is higher in anaplastic astrocytomas and glioblastomas compared with low-grade tumors and normal brain³⁸ and high levels of CatB are positively correlated with poor survival.⁹ Human glioblastoma cell lines also exhibit higher levels of CatB expression than lower grade astrocytomas¹⁰ and the functional relevance of the enzyme is confirmed by a marked reduction in in vitro invasiveness of a glioblastoma cell line transfected with antisense CatB cDNA.¹¹ There is evidence of altered trafficking of CatB in glioblastomas, with a redistribution of enzyme to the cell periphery.¹² In addition, a potential role in invasion has been suggested by high levels at the tumor periphery and in infiltrating cells.¹³ The closely related cysteine proteinase cathepsin L (CatL) has also been implicated in glioblastoma invasion in an in vitro model.¹⁴

Although much of the attention on cysteine proteinases in cancer has focused on CatB and CatL, another member of the family, cathepsin S (CatS), is also capable of degrading ECM macromolecules such as laminin, collagen, elastin, and chondroitin sulphate proteoglycan (CSPG).¹⁴ Furthermore, it is markedly more stable than either CatB or CatL at extracellular pH,¹⁵ a property that is compatible with a role in extracellular proteolysis. Cathepsin S expression has predominantly been associated with cells of monocyte-macrophage lineage. Functions ascribed to CatS include degradation of the MHC class II
chaperone II before peptide loading in the endosomal compartment and processing of the amyloid precursor protein. Secreted macrophage-derived cysteine proteinases (including CatS) are implicated in ECM remodeling in both pathological and physiological conditions. Inflammatory mediators increase CatS secretion from macrophages leading to ECM protein degradation. Growth factors such as basic fibroblast growth factor (bFGF) have been shown to up-regulate CatS expression. These properties of inducibility, capacity to degrade extracellular macromolecules, and stability at neutral pH are consistent with a role in tumor invasion. To date, expression of CatS has not been characterized in brain tumors and the current project is designed to assess the expression of CatS in human astrocytoma biopsies and cell cultures.

Materials and Methods

Cell Culture

The G-CCM cell line was initiated from a human anaplastic astrocytoma. The CB 109 and the U251MG cell lines (American Type Culture Collection, Rockville, MD) were derived from human glioblastomas multiforme. All other cell cultures were derived from brain tumor biopsy specimens from the Regional Neurosurgery Unit of the Royal Victoria Hospital and were used experimentally within 5 to 10 passages. Cultures were tested regularly for mycoplasma using the Hoechst dye-binding method and were found to be negative. Cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20 mmol/L glutamine and 10% fetal calf serum. All cell culture media and reagents were purchased from Gibco BRL, Paisley, Scotland.

Cathepsin S Assay

Cells for CatS assay were harvested by scraping from confluent T25 flasks, resuspended in 1 ml of Hanks’ balanced salt solution (HBSS) and sedimented at 1000 g for 5 minutes. The cell pellet was made up to 250 µl in MES buffer (50 mmol/L, pH 6.0) containing 0.1% TritonX-100. Complete cell disruption was ensured by sonication (MSE Soniprep150, Sanyo-Gallen-Kamp, Loughboro, Leicestershire, UK). The protein concentration of the homogenate was determined using the BCA reagent kit (Pierce) and aliquots were diluted to a concentration of 2 to 5 mg/ml and the supernatants were used for CatS assay.

CatS activity was assayed using carbobenzoxy-valyl-valyl-arginyl-N-methylcoumarylamide (VVR) as substrate. Before assay, homogenates were incubated in 100 mmol/L phosphate buffer (pH 7.5) for 1 hour at 37°C to inactivate cathepsins B and L, after which the pH was returned to pH 6.0 using 0.5 mol/L of MES buffer. Assays were performed in 96-well microtitre plates in the presence of 1 mmol/L dithiothreitol, 200 mmol/L ethylenediaminetetraacetate, and 100 µmol/L VVR in 200 mmol/L MES buffer, pH 6.0. Incubation at 37°C was continued for 90 minutes and the reaction was terminated by addition of 100 mmol/L of acetate buffer, pH 4.3. Fluorescence generated by release of methylcoumarin (nMec) from the substrate was read in Perkin Elmer LSS08 luminescence spectrophotometer (Perkin Elmer, Shelton, CT, USA) with excitation and emission wavelengths of 370 nm and 460 nm, respectively. Each assay was performed on triplicate samples and controls were included with substrate omitted from the assay. A calibration line was prepared using nMec, and CatS activity was calculated as nmol nMec produced/mg protein hour−1. There was a linear relationship between relative fluorescence activity and nMec concentration and the enzyme assay was linear with respect to reaction time and protein content of the cell homogenate.

For assay of extracellular CatS activity, confluent cultures were maintained for 24 hours in serum-free medium to obviate the effect of endogenous cysteine proteinase inhibitors in fetal calf serum. The medium was then removed from the cells and centrifuged at 1000 × g for 5 minutes to remove any detached cells before assay. Aliquots of medium (30 µl) were assayed for CatS activity using the same procedure used for cell homogenates. Extracellular CatS activity is expressed as nmol nMec produced/mg cellular protein hour−1.

Reverse Transcription-Polymerase Chain Reaction and Sequencing

DNA was prepared from confluent astrocytoma cultures and normal brain using RNAstat 60 (Biogenesis Ltd., Poole, Dorset, UK), reverse-transcribed with Maloney murine leukemia virus (MMLV) reverse transcriptase (Advanced Biotechnologies, Columbia, MD) and cDNA amplified by polymerase chain reaction (PCR). The CatS-specific primers used for PCR were designed to flank the CatS coding region and generate a 1002-bp amplicon. They correspond to positions 158–177 and 1123–1153, respectively.

CatS forward primer: 5’-atgaaacggctggtttgtgt-3’
CatS reverse primer: 5’-ctagatttctggtaaggagggtagc-3’

The 1001-bp DNA fragment was purified (Gibco) from the 1% agarose gel and ligated into pIND/V5-His-TOPO using TA Cloning (Invitrogen, UK). The resulting construct was used to transform TOP10 cells. Positive clones were selected and plasmid DNA was prepared (Concert MaxiPrep; Gibco). The fragment was sequenced using
were subjected to microwave antigen retrieval as previously described,26 with variations, using a modified Boyden chamber (Costar Transwell plates) with 12-μm pore membranes. The membranes were coated with Matrigel (100 μg/cm²) and allowed to dry overnight in a tissue culture hood; 5 × 10^5 U251MG glioblastoma cells were added to the well in 500 μl serum-free medium.

Experiments testing the effect of the CatS inhibitor, 4-Morpholineurea-Leu-HomoPhe-vinylsulphone (LHVS), a gift from Axys Pharmaceuticals, used medium containing LHVS at concentrations of 10 nmol/L and 50 nmol/L. 1.5 ml of homologous conditioned growth medium was added to the lower chamber and triplicate invasion plates were incubated at 37°C and 5% CO₂ for 24 hours. Cells remaining on the upper surface of the membrane were removed by wiping and the invaded cells were fixed in Carnoy's fixative for 15 minutes.

After drying, the nuclei of the invaded cells on the lower surface of the membrane were stained with Hoechst 33258 (50 ng/ml) in HBSS for 30 minutes at room temperature. The insert was washed twice in PBS and mounted in Citifluor for microscopic examination. Invaded cells were viewed with a Leica TCS/NT confocal microscope equipped with a krypton/argon laser.

In Vitro Invasion Assay

Invasion assays were performed as previously described,27 with variations, using a modified Boyden chamber (Costar Transwell plates) with 12-μm pore membranes. The membranes were coated with Matrigel (100 μg/cm²) and allowed to dry overnight in a tissue culture hood; 5 × 10^5 U251MG glioblastoma cells were added to the well in 500 μl serum-free medium. Experiments testing the effect of the CatS inhibitor, 4-Morpholineurea-Leu-HomoPhe-vinylsulphone (LHVS), a gift from Axys Pharmaceuticals, used medium containing LHVS at concentrations of 10 nmol/L and 50 nmol/L. 1.5 ml of homologous conditioned growth medium was added to the lower chamber and triplicate invasion plates were incubated at 37°C and 5% CO₂ for 24 hours. Cells remaining on the upper surface of the membrane were removed by wiping and the invaded cells were fixed in Carnoy's fixative for 15 minutes.

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Figure 1. RT-PCR of mRNA extracted from cultures derived from astrocytomas of World Health Organization grade I (1 to 3), World Health Organization grade II (4 to 7), World Health Organization grade III (8 and 9), and World Health Organization grade IV. 10 and 11: RT-PCR of mRNA extracted from a normal brain sample. 12: PCR, without RT step, of mRNA extracted from World Health Organization grade IV cell culture to demonstrate the absence of DNA contamination in the mRNA preparation. 13: Positive control mRNA sample of 500 bp. 14: Water (negative control). 15: Size markers. 16: CatS is represented by the fraction of 996 bp.
microscope. Four digital images of representative fields from each of the triplicate membranes were taken at a magnification of ×200. Results were expressed as the mean number of cells present per field.

Results

RT-PCR

mRNA isolated from a range of astrocytoma cell lines was reverse transcribed and subjected to PCR using primers specific for sequences flanking the coding region of CatS. Agarose gel electrophoresis (Figure 1) showed a single amplicon of a size consistent with CatS (996 bp). After purification the 996 bp amplicon was cloned, sequenced, and shown to have complete identity with the sequence of CatS (GenBank Accession Number M90696).

Immunohistochemistry/Cytochemistry

Thirty-one brain tumor biopsy specimens were examined, representing all four World Health Organization grades:28 (5 grade I, 10 grade II, 5 grade III, 11 grade IV). In all cases tumor cells were positive for CatS expression (Figure 2A and B). Normal astrocytes and other glial cells (Figure 2C), neurones (Figure 2C), neurons (Figure 2C), and endothelial cells (Figure 2B) were CatS-negative while reactive astrocytes in zones of gliosis were faintly CatS-positive (Figure 2E). The intensity of neoplastic astrocyte staining within and

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Figure 2. A: CatS immunostaining of World Health Organization grade II astrocytoma. B: CatS immunostaining of World Health Organization grade IV astrocytoma (inset shows area of endothelial hyperplasia (e) with CatS-negative endothelial cells). C: CatS immunostaining of normal brain. n, neuron; a, astrocyte; o, oligodendrocyte; m, macrophage. D: CD68 staining of tumor-associated microglia/macrophages in World Health Organization grade IV astrocytoma (inset shows perivascular macrophages). E: CatS immunostaining of brain with gliosis from a World Health Organization grade II astrocytoma (r, reactive astrocyte). Original magnification, ×400.
between grades was variable and there was no correlation between level of expression and tumor grade.

CD68 immunostaining confirmed the presence of cells of microglia/monocytic origin within tumors (Figure 2D) and in surrounding brain. Double immunohistochemical staining for CatS and CD68 revealed expression of CatS in the CD68-positive microglia/monocytic cells as well as in CD68-negative tumor cells (Figure 3, A and B). Double immunohistochemical staining for CatS and GFAP revealed distinct localization of CatS and GFAP within astrocytoma cells (Figure 3C). Twenty-one astrocytoma cell cultures examined were CatS-positive and immunostaining showed a punctate cytoplasmic distribution characteristic of lysosomal localization (Figure 3D). There was no correlation between the degree of immunostaining and grade of tumor.

**Cathepsin S Activity**

Biopsies: CatS activity in tumor lysates varied considerably between tumors (Figure 4). Astrocytoma grades I to III had

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**Figure 3.** A: Immunostaining of grade IV astrocytoma to show CD68-positive microglia/macrophages (arrows). B: CatS/CD68 dual labeling. Microglia/macrophages are positive for CatS and CD68 (arrows) whereas astrocytoma cells (arrowheads) are only positive for CatS. C: CatS (red)/GFAP (green) dual labeling. The lysosomal localization of CatS immunostaining (I) is distinct from that of GFAP which is predominantly within tumor cell foot processes (p). m, mitotic figure; n, outline of nucleus; bv, blood vessel. D: CatS immunostaining of astrocytoma cells in vitro. Nuclei are counterstained with propidium iodide. Original magnification, ×400.

**Figure 4.** Specific activity of CatS in lysates derived from biopsies of astrocytomas I-IV.
similar ranges of activity; however, while some grade IV tumors expressed activity levels similar to the lower grades, almost half of them had markedly higher levels.

Cell Cultures

Activity of CatS was measured in 31 astrocytoma cultures. Intracellular activity was present in all cultures and within each grade there was considerable variation in specific activity (Figure 5A). There was no correlation between CatS activity and grade of the tumor of origin.

Invasion Assay

The effect of the CatS inhibitor LHVS29 was assessed using the U251MG glioblastoma cell line in the Matrigel invasion assay. Incorporation of 10 nmol/L and 50 nmol/L LHVS in the medium produced significant decreases (P < 0.0001) in invasion of 49% and 61%, respectively (Figure 6).

Discussion

Biochemical and immunohistochemical studies of the cysteine proteinases CatB and CatL have reported their presence in astrocytomas in contrast to their absence from astrocytes in normal brain. The present investigation has demonstrated for the first time that the related cysteine proteinase CatS is also expressed in astrocytomas in vivo and in vitro. Normal astrocytes, however, were negative for CatS immunoreactivity in agreement with other studies of CatS in normal brain. Neurones, oligodendrocytes, and endothelial cells were also CatS-negative. Petanceska used in situ hybridization on rat brain to show that in contrast to the wide distribution of CatB and CatL, the expression of CatS mRNA is restricted to monocyte-derived microglial cells. In human brain also, CatS is not widely distributed. In an immunohistochemical study, Lemere et al demonstrated the absence of CatS from normal astrocytes, but in the brain of patients...
with Down syndrome and Alzheimer’s disease CatS was up-regulated in a subset of astrocytes and in neurones with neurofibrillary tangles.

Few studies of CatS in tumors have been carried out, but in an immunochemical survey of soft tissue sarcomas Wurl et al. showed that 65% of cases expressed CatS. CatS expression was associated with poor prognosis and local recurrence. Fernandez et al. demonstrated CatS expression at all stages of prostatic carcinoma progression with no correlation between expression levels and tumor grade. In a study of lung cancer, Kos et al. found higher levels of CatS protein in tumor tissue when compared with adjacent control tissue, but in this case the risk of death was higher in patients with low levels of CatS in the tumor and parenchyma. Nissler et al. demonstrated that intracellular pro-CatS in a lung tumor cell line has twice the half-life of pro-CatS expressed in human macrophages and that substantial amounts of pro-CatS were secreted in vitro. The present immunohistochemical investigation of CatS protein found up-regulated CatS expression in astrocytoma cells but did not suggest a positive correlation between CatS expression level and astrocytoma grade. However, direct measurement of CatS activity in astrocytoma homogenates indicated highest activity levels in grade IV tumors. Interpretation of CatS expression data from tumor lysates is problematic because of the cellular complexity of the tumor environment, which contains host normal and inflammatory cells in addition to tumor cells. We have shown that normal glial cells, neurones, and endothelial cells do not express CatS. However, macrophages and microglia may make significant contributions to the apparent CatS expression of the tumor. CD68/CatS dual labeling in the current investigation confirms the presence of considerable numbers of CatS-positive microglia/macrophages. Astrocytomas characteristically have a high content of cells of microglial/monocytic origin, with a higher density present in high-grade astrocytomas relative to low-grade tumors. A positive correlation has been reported between microglia/macocyte content and astrocytoma grade and this may explain the high CatS-specific activity that we found in many grade IV tumors.

It seems possible that brain microglia and macrophages may play an active role in tumor invasion rather than non-specific reaction to tissue injury. This is suggested by Bettinger et al. who have shown that microglial cells promote astrocytoma migration in vitro. The secreted migration-stimulating factors were not specifically identified, although growth factors such as transforming growth factor-β were suggested as potential candidates. However, microglia/macrophages express high levels of a range of proteolytic enzymes including CatS which may augment the proteolytic activity of the tumor cells and promote invasion by modifying the extracellular matrix.

To assess tumor cell expression and a potential role in invasion in the absence of other cell types, we have used astrocytoma cultures. We found no correlation between intracellular CatS activity and grade of the tumor of origin. In contrast, extracellular activity, which is likely to be most relevant to proteolysis associated with the invasive process, was significantly elevated in grade IV tumors. The absence of up-regulation of intracellular enzyme in the presence of increased secretion may be unexpected, but a similar situation has been described during macrophage activation. Liuzzo et al. found that lipopolysaccharide and other inflammatory mediators induced an increase in CatS secretion while expression of intracellular CatS mRNA and protein was down-regulated.

Inhibition of CatS activity of U251MG cells with LHVS resulted in a 60% reduction in invasion, suggesting a significant role for CatS in the invasive process. Biroc et al. observed a comparable 60% reduction in joint tissue/ECM destruction with oral LHVS administration in a rat model of rheumatoid arthritis. Local invasion of tumor cells beyond resection borders is a feature of malignant astrocytomas leading to recurrence which is largely responsible for failure of conventional astrocytoma therapy. Protease inhibitors may therefore offer a method for the control of proteolytic activity associated with ECM degradation and cell invasion. It is likely that successful treatment of malignant astrocytoma will involve the application of combinatorial therapeutic strategies.

CatS retains optimal activity over a wider pH range (5.0 to 7.5) than CatB and CatL (5.5 to 6.5). The broad pH profile of mature CatS indicates that it would remain active in the astrocytoma extracellular environment. We have demonstrated the presence of extracellular CatS activity in vitro which may reflect either secretion of mature enzyme or extracellular activation of secreted proenzyme by other proteinases. Active CatS in the extracellular environment of astrocytomas may contribute to degradation of ECM components thereby facilitating the invasive process.

In summary, this investigation has demonstrated that CatS is expressed and active in astrocytoma cells. In addition, we have provided evidence that CatS plays a role in the invasive process and is therefore a potential target for anti-invasive therapy.

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References

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