Immunohistochemical expression of Cathepsin H protein in Glioblastoma Multiforme: Diverse subcellular localization patterns suggest a complex role in the biology of these tumors

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Citation

Abstract

Patients diagnosed with Glioblastoma Multiforme (GBM) usually survive less than a year, even with the latest treatment protocols, thus there is an urgent requirement for identifying molecular markers that could be utilized for targeted therapy. The lysosomal proteases are believed to contribute to the invasive process of tumors by breakdown of extracellular matrix. Cathepsin H is a lysosomal protease whose expression has not been fully characterized in GBM. We examined the pattern of CH protein expression immunohistochemically in 24 GBM, 18 other brain tumors and 3 non-tumor cases. CH was expressed in all cases of GBM, although there was considerable heterogeneity within each tumor. Several patterns of CH expression were observed including granular and diffuse cytoplasmic staining. The diversity in CH expression patterns in GBM indicates a complex role for CH in the biology of these tumors, and warrants further investigation for developing a novel molecular target for therapy.

INTRODUCTION

Glioblastoma multiforme (GBM) is one of the most aggressive neoplasms and has a particularly bad prognosis even when the latest treatment protocols are employed. Craniotomy with surgical excision of the tumor may provide temporary alleviation of symptoms due to release of intracranial pressure, however, surgery may not be curative alone as re-growth of the tumor usually occurs rapidly. The reason for failure is infiltration of tumor cells beyond the gross visible confines of the tumor, thus reducing the role of surgery to debulking of the tumor. Radiation may provide benefit only early in the course of treatment since tumors often acquire radio-resistance. Chemotherapy has limited utility since GBM most commonly afflicts elderly populations who are particularly vulnerable to chemo-toxicity. Thus, in the hope of reducing morbidity and improving the efficacy of treatment, targeted therapy through tumor-associated molecular markers is gaining importance.

Infiltration by tumor cells into surrounding tissues is an active process associated with increased release of proteolytic enzymes that modify the host extracellular matrix. The lysosomal cathepsins are believed to contribute to this invasive process by breaking down structural extracellular components [1]. In brain tissues such proteolytic activity may assist in the dissolution of dendritic and glial processes that form the intricate network of the brain parenchyma. Previous studies have examined the expression of cathepsins including CH in GBM [2,3,4], but to our knowledge, the pattern of cathepsin H (CH) protein expression in GBM has not been fully characterized by immunohistochemistry. The aim of the present study was to examine the expression of CH in GBM by immunohistochemistry and to characterize in detail its subcellular distribution in a variety of GBM. Also, CH expression in GBM is compared to its expression in normal (and control) brain tissue, high and low grade gliomas, meningiomas and metastatic carcinomas.

MATERIALS AND METHODS
TISSUE SPECIMENS

Archival paraffin-embedded tissue specimens were obtained from the Department of Pathology, at University of Florida, Jacksonville Campus, Jacksonville, FL. We studied tissue

from 45 patients. These included 42 tumor cases, comprised of 24 GBM, 3 high-grade gliomas, 4 low-grade gliomas, 4 meningiomas, 6 metastatic carcinomas to brain and 1 medulloblastoma. Several cases of GBM also had normal appearing (control) cortex present in histological sections. Furthermore, sections from 3 temporal lobectomies (removed for seizures and with no tumor) were stained for CH as additional control tissue. Pathology reports were available with histological categorization of brain tissue specimens on hematoxylin and eosin stained sections according to the WHO classification system ($_5$).

IMMUNOHISTOCHEMISTRY

Immunohistochemical evaluations were performed using standard protocols. Tissue sections (4 microns) were mounted on charged glass slides, and loaded on to Ventana Benchmark XT instrument (Ventana, Tucson, AZ) for automated immunostaining including baking, de-waxing, rehydration, blocking of endogenous peroxidase, incubation in primary and secondary antibodies, label / color developer and Tris buffer washings. Primary rabbit polyclonal anti-human cathepsin H antibody was obtained from Athens Research Laboratories (Athens, GA) and applied at a dilution of 1: 2,000 for 2 h at 37 C. All other reagents were obtained from Ventana. A positive control specimen that contained regions of previously evaluated astrocytic tumor and normal brain cortex was always included in the test batch. A negative control was provided by exclusion of primary antibody from the protocol. Immunohistochemically stained sections were reviewed and scored for percentage of tumor cells staining positive for CH (visual estimation by S Shuja and S Goodison), and subcellular distribution patterns such as type of granules (fine or coarse) and other distinct patterns of staining. The percentages of positive tumor cells were assessed in 'hot-spots', i.e. the regions of highest expression of CH (intensity and number of positive cells) on histological evaluation of each case. The intensity of CH positive staining was recorded as a score of +1 to +3. ($_6$,$_7$). To normalize the intensity of staining across specimens and batches, a score of +3 was given to strongly staining endothelial cells in a given batch.

RESULTS

CATHEPSIN H STAINING IN CONTROL BRAIN TISSUES

Control brain tissue did not show any appreciable CH staining (Figure 1A), however, in some sections occasional astrocytic cells exhibited fine granular staining (intensity +1)

(Figure 1B). We also used brain tissues surgically removed from patients who had presented with seizures unassociated with tumor, as a control. Examination of these control cortices from temporal lobectomies revealed no appreciable expression of CH in cortex except for some granular staining (intensity +2) observed in oligo-like cells in the white matter (not shown). It is notable that in 2 of these 3 temporal lobectomy specimens, and in some control brain cortices of GBM, CH staining revealed few plaque-like structures ranging in size from 0.06 to 0.1 mm (Figure 1C). A consistent feature in control cortices from GBM cases and from temporal lobectomies was coarse granular staining (intensity +3) in endothelial cells lining the vasculature [Figure 1b].

CATHEPSIN H EXPRESSION IN GLIOBLASTOMA MULTIFORME

CH expression was observed in all 24 GBM cases examined. The intensity of staining in neoplastic astrocytic cells was usually noticeably stronger than that of astrocytes present in the non-neoplastic brain tissue. There was considerable heterogeneity of CH expression, with different regions of tumor either staining negatively or positively for CH. Within positively staining regions we estimated a maximum percentage of CH expressing tumor cells in each individual tumor and this ranged from less than 5% to over 90%. The tumors that showed a higher percentage of positive cells, also showed higher staining intensity. When greater than 50% of tumor cells were positive, 10 of 12 tumors exhibited staining intensity of either +3 or +2 (Table 1). Conversely, when less than 50% tumor cells were positive for CH, 10 of 12 tumors exhibited staining intensity of either +1 or +2. Overall, 50% of GBM exhibited +3 staining when >50% of tumor cells were positive, but in the fraction of tumors with less than 50% cells positive for CH, only 16% exhibited +3 staining. Distribution of CH was not limited to a particular cell shape or size. CH was expressed in all types of component cells, including large and small cells, multinucleated cells or lipidized cells, only in two cases the large cells in the tumor showed increased CH expression while the small cell component was negative.

Figure 1

Table 1: Relationship between the intensity of CH immunohistochemical detection and percentage of positive tumor cells in glioblastoma multiforme.

% CH-positive tumor cells	Staining intensity		
	+3	+2	+1
>50%	6/12	4/12	2/12
<50%	2/12	5/12	5/12
All GBM cases	8/24	9/24	7/24

DIVERSE SUBCELLULAR PATTERNS OF CATHEPSIN H EXPRESSION IN GLIOBLASTOMA MULTIFORME

CH expression was detected in individual malignant astrocytes of GBM in various subcellular patterns. The two common patterns were a granular pattern and a diffuse cytoplasmic staining pattern. The granular staining was a mixture of fine and coarse granules randomly distributed in the cell cytoplasm (Figure 1D-F). Several additional patterns of staining were also observed which commonly as a combination of two or more patterns in a particular tumor, although rarely, these existed as a single dominant pattern. The various patterns are described as follows:

Golgi-type pattern: Fine and coarse granules concentrated in the cytoplasm close to one pole of the nucleus (Figure 1G); Perinuclear pattern: Fine and coarse granules surrounding the nucleus (Figure 1H); Glial mesh-type pattern: composed of fine and coarse granules, as well as "clumps" in the fibrillary matrix remote from the main cytoplasmic body of the neoplastic cells (Figure 1J); Axial-type pattern: a concentration of fine and coarse granules around a central axis in the cytoplasm of the cells (possibly around a major glial process) was seen in one case (Figure 2A); Peri-axonal pattern: residual axonal processes within the tumor sometimes appeared to be highlighted due to CH staining along their length in a lace-like fashion (Figure 2B).

The combination of the Golgi-type and perinuclear patterns were the most frequently observed patterns after the combination of granular and diffuse cytoplasmic patterns of CH expression in GBM. The various subcellular staining patterns did not appear to have any obvious association with intensity of staining in individual tumors, although the Golgi-type and perinuclear pattern was usually +2 or higher in intensity.

HIGH AND LOW-GRADE GLIOMAS

We examined 3 cases of high-grade gliomas and 4 cases of low-grade glioma. There was considerable heterogeneity in the percentage of positive cells from tumor to tumor and within the same tumor. The low-grade gliomas showed a predominantly diffuse cytoplasmic staining pattern (intensity +1, +2), (Figure 2C) whereas the high-grade gliomas mostly exhibited Golgi and perinuclear patterns of staining. (Figure 2D). The vascular endothelial proliferations in high and low grade gliomas expressed high levels of CH protein (intensity +3).

CATHEPSIN H STAINING IN ENDOTHELIAL CELLS OF GLOMERULOID VASCULAR PROLIFERATIONS IN GBM

Glomeruloid vascular endothelial proliferations in GBM showed granular staining pattern in endothelial cells, however, not all endothelial cells lining the lumen of a capillary were positive for CH. In some tumors, a linear pattern of CH staining in endothelial cells was observed, while in others both diffuse cytoplasmic and granular staining patterns were seen. The intensity of staining was generally +2 to +3 (Figure 2E).

CATHEPSIN H STAINING IN TUMOR INFILTRATING MACROPHAGES IN GBM

Macrophages infiltrating GBM were always strongly positive for CH (+3 staining intensity), with usually coarse granular staining pattern exhibited in the cell cytoplasm. This is consistent with the reported location of CH within the lysosomes of macrophages.

MEDULLOBLASTOMA

In one case of medulloblastoma there was no appreciable CH expression in the majority of neoplastic cells, however, focal areas (presumably more differentiated cells) expressed strong (intensity +3) diffuse cytoplasmic or Golgi-type patterns of staining [Figures 2F-G]. The endothelial cells also showed granular positive staining for CH.

MENINGIOMAS

In the four meningiomas examined CH expression was seen in less than 10% of tumor cells, among these the low-grade meningiomas demonstrated less than 5% positive cells. The pattern of CH expression was a mixture of granular, Golgi and perinuclear types with an intensity of +2 (Figure 2H). In contrast to that observed in gliomas, it was noticeable that the endothelial cells in meningiomas usually displayed little
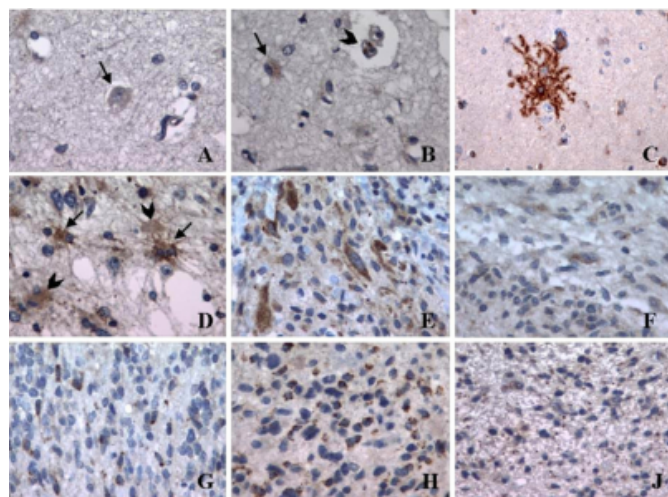
or no CH expression.

METASTATIC TUMORS

A total of six metastatic tumors to the brain were examined. Two metastases from neuroendocrine carcinomas exhibited no appreciable CH expression in the tumor other than in the endothelial cells and macrophages. Three cases of metastatic adenocarcinomas (from lung, colon and one from an unknown primary origin) showed 5% to 50% of tumor cells expressing CH (intensity +1) with a diffuse cytoplasmic and/or granular pattern in the apical portions of the cytoplasm or accentuated along the basement membrane (Figure 2j). A metastatic thymic carcinoma exhibited strong (intensity +3) diffuse cytoplasmic staining (not shown).

Figure 2

Figure 1: Cathepsin H expression in human brain tissues and Glioblastoma multiforme.
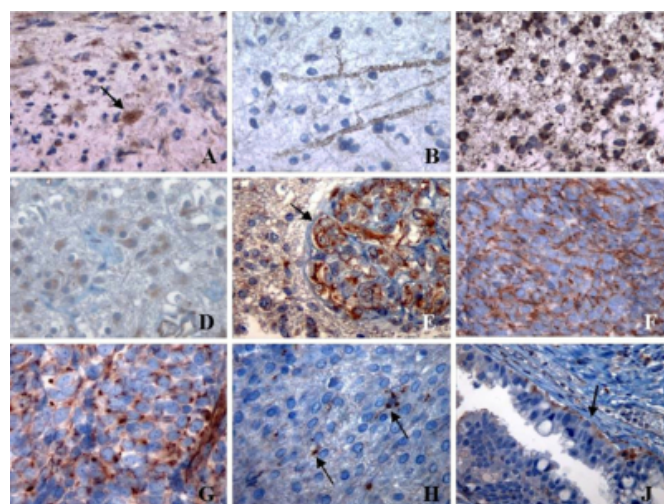

Cathepsin H (CH) protein expression (brown staining) in human brain tissues is shown, as detected immunohistochemically on formalin-fixed, paraffin-embedded tissue sections. (A), Control cortex from a patient with GBM: A neuron with Nissl's substance (arrow) with no appreciable CH staining; (B), Control cortex from a patient with GBM: fine and coarse granules (intensity +1) in an astrocytic cell (arrow). CH staining (intensity +3) is evident in endothelial cells lining a capillary (arrowhead); (C), Immunohistochemical staining of CH showing a 'plaque' in a control cortex from a temporal lobectomy specimen. Plaques detected ranged from 0.06 to 0.1 mm (maximum diameter); (D), GBM (lysosomal pattern); fine & coarse granules in the cytoplasm of neoplastic cells (intensity +3, arrows). Diffuse cytoplasmic staining (intensity +1) is also evident (arrowheads). A number of microcysts can be seen at

bottom right; (E), GBM (lysosomal pattern). Large neoplastic cells appear to be 'bags of protease'; (F), GBM (diffuse cytoplasmic pattern); most cells are showing diffuse cytoplasmic staining (intensity +1). Some granular staining is also observed in some cells. Pseudopalisading necrosis is seen at bottom left; (G), GBM (Golgi-type pattern). Coarse granular staining concentrated close to one pole of the nucleus. Fine granular staining in the cytoplasm is also seen in some cells; (H), GBM (Perinuclear-type pattern); CH coarse granular staining is seen concentrated in the perinuclear region of the cytoplasm (intensity +3); (J), GBM (Glial-type pattern); Coarse granules are randomly scattered in the glial network (intensity +3). These granules appear to be associated with dendritic processes and thus lie far away from the main cell body.

Figure 3

Figure 2: Cathepsin H expression in Glioblastoma Multiforme and other brain tumors.

Cathepsin H (CH) protein expression (brown staining) in human brain tissues is shown, as detected immunohistochemically on formalin-fixed, paraffin-embedded tissue sections. (A), GBM (Axial-type pattern); A concentration of fine and coarse granules around a central axis (arrow) in the cytoplasm of the cells (possibly around a main dendritic process); (B), GBM, (Periaxonal-type pattern). Residual axonal processes appear to be highlighted by CH staining in a lattice-like framework running along the length of the processes; (C), High-grade mixed oligo/astrocytoma, WHO Grade III (Perinuclear-type and Glial-type patterns) demonstrates staining (intensity +3); (D), Low grade glioma (pilocytic astrocytoma, WHO grade I). Diffuse cytoplasmic staining (intensity +1) for CH in neoplastic astrocytes; (E), Glomeruloid capillary endothelial

proliferation in GBM showing fine granules and diffuse cytoplasmic staining (intensity +3) for CH in endothelial cells (arrow); (F), Medulloblastoma showing diffuse cytoplasmic staining (intensity +3) for CH in neoplastic cells; (G), Medulloblastoma showing Golgi-type pattern; (H), Meningioma. The majority of cells exhibit no staining, but a few cells show a Golgi-type and perinuclear CH expression pattern (arrows); (J), Moderately differentiated metastatic adenocarcinoma. There is prominent expression of CH in the basal part of the cell cytoplasm, with accentuation along the basement membrane (arrow).

DISCUSSION

Cathepsins are lysosomal proteases, which are expressed in most human cells. Increased levels of cathepsins have been reported in a number of tumor types including colon, thyroid, urinary bladder, breast and skin ([6,7,8]). Such increased levels of cathepsins are thought to assist malignant cells in degrading basement membrane and extracellular matrix components ([1,9]), thus assisting in the processes of invasion and metastasis. Cathepsins have also been studied to some extent in brain tumors. Increased expression of CB has been demonstrated in GBM and other tumors of astrocytic origin ([2]), and increased cathepsin L activity has been described to be a significant factor contributing to the invasive potential of human brain tumor cell lines ([3]). In addition, increased levels of CH enzyme activity have been reported in GBM and other astrocytic tumors, with an overall trend of increased enzyme activity through the increasing grades of these tumors ([4]).

The specificity of the CH antibody used for our immunohistochemical study was confirmed by western blotting, as described previously ([7]). Four types of purified human liver cathepsins, including cathepsins B, L, D and H were run side by side on a single western blot and probed simultaneously with the cathepsin H antibody which detected only purified human cathepsin H protein on this blot. Our immunohistochemical study with this CH antibody demonstrated that in the non-neoplastic (control) brain, occasional scattered astrocytic cells and some endothelial cells may express CH. The control brain also exhibited few "plaque-like" structures (Figure 1C), which may represent a particular cell type or deposition of extracellular material containing CH protein. The nature and significance of these structures need be determined. Our most significant finding was that CH was expressed in all 24 GBM cases examined, and showed several patterns of subcellular distribution of

CH. The predominant patterns of staining were granular (fine and coarse granules), and diffuse cytoplasmic pattern, which often coexisted. The presence of randomly distributed fine and coarse granules in the cytoplasm of astrocytic cells suggested localization of this protease in the lysosomes. On the other hand, diffuse cytoplasmic staining may suggest localization of CH to the plasma membrane or cytosol. Indeed, relocalization of cathepsins to plasma membrane has been reported in various other tumors ([10]). Furthermore, some of the CH relocated to the plasma membrane or cytosol may be secreted into the extracellular milieu, resulting in proteolytic cleavage of matrix macromolecules in the extracellular environment ([11]).

After the predominant patterns of combined granular and diffuse cytoplasmic staining, the next most common patterns observed were a combination of Golgi-type and perinuclear patterns. The Golgi-like pattern of accumulation of CH in GBM may be a manifestation of a rate-limiting event in the processing and passage of CH to its targeted cellular compartment. Being a lysosomal protease CH is synthesized as a preproprotein in the endoplasmic reticulum (ER) [12]. During its passage through the ER the prepeptide is removed and the residual propeptide signal targets it to the Golgi apparatus where it is glycosylated, phosphorylated and disulfide bridges are formed. The tumors that exhibited Golgi-type pattern did not generally express significant granular or diffuse cytoplasmic staining, suggesting a relative block in CH transportation at the level of Golgi in these tumors. In the glial mesh pattern, CH was randomly distributed in the glial network as fine or coarse granules or 'clumps.' The granules observed in the glial mesh may be highlighting accumulation of cellular CH at anatomic boundaries between cells such as cell-cell synapses. The axial pattern of CH expression observed in one case suggested a central axis such as a main glial process around the base of which fine and coarse granules accumulated, thus highlighting its origin from the cell body (Figure 2a). This pattern was also associated with a glial mesh pattern. An axonal-type CH expression pattern was also seen in one case, with the residual axonal processes in the tumor being highlighted by CH as a lattice-like framework running along the length of the residual axons (Figure 2B). It is possible that such an association of CH, and possibly other proteases, precedes the dissolution of axons in the area infiltrated by tumor.

The high expression of CH in capillary endothelial

proliferations in GBM suggests an important role for CH in neovascularization. Inhibition of a related cysteine protease, cathepsin B, has been shown to suppress glioblastoma-induced neovascularization ([13]). The patterns of CH expression in capillary endothelial proliferations in GBM were variable and included presence of a coarse granules in a few cells similar to that seen in control cortex capillaries or enhanced linear staining along the capillary lumens, or presence of numerous fine and coarse granules or diffuse cytoplasmic staining (intensity +3) (Figure 2C) or any combination thereof. Increased CH may help the endothelial cells to breakdown laminin and other scaffolding around pre-existing capillaries so that new capillaries can be formed by proliferating endothelial cells.

CH was also expressed in metastatic carcinomas to the brain, meningiomas and one case of medulloblastoma studied. Further study is warranted to characterize the expression and role of CH in these tumors. Nonetheless, our demonstration of increased CH in 66% of GBM (staining intensities of +2 to +3), suggests that GBM is likely to respond to strategies aimed at inhibiting CH, thus indicating a potential avenue for developing novel therapeutic strategies which may be complementary to radiation and chemotherapy. Increased expression and diversity in the pattern of CH expression in GBM is likely a reflection of the potential functions that cathepsin H may perform in GBM, which could invasion, cell cycle regulation, modification of growth factors, apoptosis, angiogenesis, and even transcriptional regulation, such functions are being increasingly linked to cathepsins in malignancy ([14,15]).

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