Localization of gelatinase-A and gelatinase-B mRNA and protein in human gliomas

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Malignant gliomas maintain a poor prognosis and survival rate due to their marked local invasive growth and neovascularization. Matrix metalloproteinases (MMPs) have been implicated in glioma invasion and angiogenesis, but it is unknown whether they are produced by the tumor cells or surrounding stroma. Using in situ hybridization and immunohistochemistry, we found expression of mRNA for both gelatinase-A (MMP2) and gelatinase-B (MMP9) localized to tumor cells and vascular structures in glioma sections. Gelatinase-A protein expression was detected most prominently in tumor cells, with very little signal seen in vasculature. Gelatinase-B protein expression was prominent in vascular structures but was also expressed in tumor cells. Our data show that these proteases are produced by glioma cells and vascular structures and suggest that synthetic MMP inhibitors might be useful in this disease. *Neuro-Oncology* 2, 145-150, 2000 (Posted to *Neuro-Oncology [serial online]*, Doc. 99-49, April 20, 2000. URL <neuro-oncology.mc.duke.edu>)

Invasion and neovascularization for a variety of cancers depend on the degradation of the ECM² and vascular basement membranes by secreted proteases called MMPs (Azanvoorian et al., 1993; Heppner et al., 1996; Rao et al., 1993). MMPs are zinc-dependent prote-

olytic enzymes responsible for ECM degradation in many physiological and pathological tissue remodeling processes (Woessner, 1991). Two members of the MMP family, gelatinase-A (MMP2) and gelatinase-B (MMP9), together known as the type IV collagenases, are thought to play a critical role in tumor pathophysiology (Rao et al., 1993). The degradation of type IV collagen, a primary constituent of basement membranes and the major substrate for gelatinase-A and gelatinase-B, suggests the importance of these proteases in angiogenesis and tumor cell invasion. Surprisingly, in systemic cancers, it is usually the stromal cells and not the tumor cells that overexpress and secrete these proteases (Heppner et al., 1996; Nielsen et al., 1997; Zedenius et al., 1996).

The two hallmarks of malignant gliomas are their invasiveness and vascularity. Invasive glioma cells migrate beyond the main tumor mass and render these surgically incurable (Kelly et al., 1987). Gelatinase-A or gelatinase-B are commonly overexpressed in glioma cell lines and surgical specimens (Forsyth et al., 1998, 1999; Nakagawa et al., 1996; Nakano et al., 1995; Rao et al., 1993, 1996; Rutka et al., 1995; Sawaya et al., 1996; Uhm et al., 1996), but their precise function in gliomas is unknown. An understanding of their mRNA and protein localization in gliomas would help determine their functions in vivo and allow for rational targeting of MMP inhibition as a novel glioma treatment.

We used RT-PCR and zymography to characterize the expression levels of gelatinase-A and gelatinase-B. We used in situ hybridization and immunohistochemistry to determine the localization patterns of gelatinase-A and gelatinase-B mRNA and protein expression in a common set of human gliomas spanning the spectrum of glioma malignancy.

Received 9 September 1999, accepted 2 February 2000.

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²Abbreviations used are as follows: ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBM, glioblastoma multiforme; MMPs, matrix metalloproteinases; RT-PCR, reverse transcriptase-polymerase chain reaction.

Materials and Methods

Tissue Collection

Glioma specimens were collected in the operating room during surgery. A signed informed consent was given by each patient, and this study was approved by the Institutional Ethics Board of the University of Calgary. Confirmation of the histologic diagnosis was done by a neuropathologist (N.B.R.). Histologically normal brain was obtained during surgery for other causes (patients undergoing surgery for non-brain tumor diseases). The following tissues were studied for in situ hybridization and immunohistochemistry: 3 GBMs, 2 anaplastic astrocytomas, 2 low-grade gliomas, and 2 normal brain specimens. For RT-PCR and zymography, the following tissues were studied: 19 GBMs, 7 anaplastic astrocytomas, 5 malignant oligodendrogliomas, 8 low-grade gliomas, and 7 normal brain specimens.

RT-PCR

RT-PCR was performed according to the method described by Forsyth et al. (1999). Briefly, total cell RNA extraction was done using the acid guanidinium isothiocyanate method. One microgram of total RNA, determined by a GeneQuant spectrophotometer (Pharmacia, Baie d'Urfe, Quebec, Canada), was reverse transcribed to cDNA in a reaction mixture containing all appropriate deoxynucleoside triphosphates (dNTPs) and MuLV reverse transcriptase (Gibco BRL; Life Technologies, Burlington, Ontario, Canada). PCR reactions were performed with 2 µl of reverse transcriptase product serving as template DNA (synthesized cDNA), appropriate dNTPs, and 20 pmol each of the appropriate 5' and 3' target primers. *Taq* DNA polymerase (Gibco BRL) was added during the first denaturation step ("hot start" technique), and 20 pmol of 5' and 3' GAPDH primers were added at the appropriate cycle according to the "primer dropping" method (Wong et al., 1994). Primer sequences were as follows:

Gelatinase-A

5'-primer=5'-GGCCCTGTCACTCCTGAGAT

3'-primer=5'-GGCATCCAGGTTATCGGGGA

Gelatinase-B

5'-primer=5'-TGCAACGTGAACATCTTCGACGC

3'-primer=5'-TCCTCAAAGACCGAGTCCAGCT

GAPDH

5'-primer=5'-CGGAGTCAACGGATTTGGTCGTAT

3'-primer=5'-AGCCTTCTCCATGGTGGTGAAGAC

The thermocycling conditions were as follows: a heat denaturation step (94°C for 1 min), a primer-annealing step (55°C for 30 s), and a polymerization step (72°C for 1 min). Gelatinase-A was run for 29 cycles and gelatinase-B for 33 cycles. GAPDH primers were introduced for the last 23 cycles. These operative cycle numbers were established by an initial "cycle test" to allow for detection of amplification products in the exponential phase of the PCR reaction. PCR product aliquots, equalized for GAPDH signal (an internal mRNA control), were electrophoresed through 2% agarose gel containing ethidium

bromide for visualization. Computerized densitometric scanning of the images using a Hewlett-Packard Scan Jet IIc Scanner, Deskscan II software, and the public domain NIH Image program was done to analyze relative signal intensities. Data for each set were averaged and plotted on graphs for presentation.

Zymography

Zymographic analysis was performed as described previously (Forsyth et al., 1999). Briefly, 10 mg of total protein, extracted from frozen tissue by a homogenization technique, was electrophoresed through 10% denaturing sodium dodecyl sulphate polyacrylamide gels containing 1 mg gelatin substrate. Gels were incubated at 37°C to allow for renaturation and were appropriately stained. The proteases were visualized as clear bands against a Coomassie blue-stained background. Densitometric scanning analysis was performed as described above.

In Situ Hybridization

Antisense riboprobes were prepared as described previously (Forsyth et al., 1999) and labeled with digoxigenin-labeled UTP (Boehringer Mannheim; Roche, Laval, Quebec, Canada) following the manufacturer's instructions. In situ hybridization was performed as described previously (Harvey et al., 1995; Leco et al., 1997). Formalin-fixed paraffin-embedded tissue sections were hybridized at 60°C overnight. Sections were blocked in 4% normal goat serum and incubated in monoclonal mouse antidigoxigenin antibody (Boehringer Mannheim) overnight at 4°C. A horseradish peroxidase-conjugated goat antimouse antibody (Pierce, Rockford, IL) was used as secondary antibody. Gelatinase-A and gelatinase-B transcripts were localized by revealing peroxidase activity using 3,3'-diaminobenzidine kit for peroxidase (DAB Substrate Kit; Vector Laboratories, Burlington, Ontario, Canada). Negative controls involved incubation with sense riboprobes. Gill hematoxylin nuclear staining was used as a counterstain.

Immunohistochemistry

For immunolocalization of gelatinase-A and gelatinase-B, an indirect immunoperoxidase method was used. In brief, formalin-fixed paraffin-embedded tissue sections were reacted with primary antibody and incubated with a horseradish peroxidase-conjugated secondary antibody. The antibodies used for gelatinase-A were polyclonal rabbit antigelatinase-A antibody (Chemicon International, Temecula, CA); for gelatinase-B, polyclonal rabbit antigelatinase-B antibody (Cambio, Cambridge, UK); and for secondary antibody incubation, a horseradish peroxidase-conjugated goat antirabbit antibody (Pierce). Primary antibody incubation was done at 4°C overnight. Secondary antibody incubation was done at room temperature for 60 min. Gelatinase-A and gelatinase-B were localized by revealing peroxidase activity. Omission of primary antibody served as a negative control for each specimen. Gill hematoxylin nuclear staining was used as a counterstain.

Fig. 1. Quantification of gelatinase-A and gelatinase-B mRNA in human gliomas by reverse transcriptase-polymerase chain reaction (RT-PCR). A. PCR amplification of cDNA prepared from cells demonstrating the presence of transcripts for the matrix metalloproteinases (MMPs) gelatinase-A and gelatinase-B. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal intensity, an internal mRNA control, was equalized to ensure comparative validity. B and C. Relative intensities determined by densitometry were compared to the average of two control samples and were plotted in arbitrary units. Correlation between normal specimens and the various tumor grades were determined for gelatinase-A (B) and gelatinase-B (C). Error bars represent SEM. Abbreviations: LG, low grade glioma; MO/MA, malignant oligodendroglioma or malignant astrocytoma; GBM, glioblastoma multiforme.

Statistical Analysis

Quantitative expression of gelatinase-A and gelatinase-B with respect to transcript intensity from RT-PCR and lysis per mg of protein from the zymograms was compared using the Kruskal-Wallis test. Correlation of quantitative expression to glioma malignancy was done using Spearman rank correlation coefficient.

Results

Analysis of Gelatinase-A and Gelatinase-B mRNA Expression by RT-PCR

Both gelatinase-A and gelatinase-B were expressed at very low levels in normal tissues, whereas a consistent overex-

pression of these proteases was found in glioma samples (Fig. 1A). Expression of gelatinase-A in tumor tissue was variable and did not correlate with the degree of malignancy ($r = 0.2024$, Spearman's rank correlation coefficient; $P = 0.2185$) (Fig. 1B). Expression of gelatinase-B was somewhat variable; however, it did correlate more closely with glioma malignancy ($r = 0.4453$; $P = 0.0068$) (Fig. 1C).

Analysis of Gelatinase-A and Gelatinase-B Enzyme Activity by Zymography

Low levels of gelatinase-A and gelatinase-B activity were observed in the control specimens and in low-grade gliomas. Progelatinase-A (72 kDa) showed highly variable levels of activity within each tumor type; however, there was correlation with degree of tumor malignancy ($r = 0.409$; $P = 0.006$) (Fig. 2A). High levels of progelatinase-B (92 kDa) activity were seen in more malignant gliomas, showing highest activity in GBMs ($r = 0.354$; $P = 0.018$) (Fig. 2B). In two GBMs, we observed faster migrating bands of gelatinolytic activity, seen at approximately 83 kDa, that we identified as the activated form of gelatinase-B. No bands corresponding to active species were seen for gelatinase-A.

Localization of Gelatinase-A by In Situ Hybridization and Immunohistochemistry

Gene transcripts for gelatinase-A in normal brain tissue sections were observed in neurons (Fig. 3A). In glioma sections, gelatinase-A mRNA expression was observed in tumor cells and in vascular structures (Fig. 3B–3D). No detectable gelatinase-A protein expression was observed in normal brain sections (Fig. 3E). In tumor sections, this protease was prominently expressed in tumor cells, with faint signal observed in the vasculature (Fig. 3F–3H). No significant difference was seen in the distribution of gelatinase-A mRNA or protein expression among the histologic grades. Negative controls for each tissue displayed no staining (data not shown).

Localization of Gelatinase-B by In Situ Hybridization and Immunohistochemistry

Gene transcripts for gelatinase-B in normal brain sections were detected in neurons with faint signal seen in blood vessels (Fig. 4A). In gliomas, gelatinase-B mRNA was consistently expressed in the tumor vasculature and in tumor cells (Fig. 4B–4D). No detectable gelatinase-B protein expression was seen in normal brain sections (Fig. 4E). Protein expression was pronounced in blood vessels (Fig. 4F–4H), localizing to areas around the lumen of both large and small blood vessels. Localization of gelatinase-B to tumor cells was also observed in the glioma sections. No significant difference in the distribution of gelatinase-B mRNA or protein was observed among the histologic grades. Negative controls showed no staining (data not shown).

Discussion

Fig. 2. Quantification of the average zymographic activity for normal brain tissue and brain tumor tissue specimens. Relative intensities determined by densitometric analysis were plotted in arbitrary units. Correlations between normal specimens and samples of various tumor grades were established for (A) gelatinase-A and (B) gelatinase-B. Error bars represent SEM. See Fig. 1 for definitions.

To our knowledge, this study is the first to use in situ hybridization and immunohistochemistry to combine the localization of gelatinase-A and gelatinase-B in a common set of glioma specimens spanning the spectrum of glioma malignancy. Others have reported the expression of either gelatinase-A (Costello et al., 1994; Sawaya et al., 1996) or gelatinase-B (Ashida et al., 1996; Rao et al., 1996). An increased level of mRNA expression for both proteases versus normal brain was seen for glioma specimens analyzed by RT-PCR. Zymographic analysis showed an increased level of gelatinolytic activity from the latent forms of both enzymes in glioma specimens compared with normal brain. Both gelatinase-A and gelatinase-B mRNA localized to the tumor cells and vascular structures. Most of the gelatinase-A protein expression was in tumor cells, with only faint signal observed in blood vessels. Gelatinase-B maintained a pronounced protein expression in blood vessels, with tumor cells also expressing protein. These data suggest that both gelatinase-A and gelatinase-B are produced by glioma cells and are potentially important in gliomas. Due to the presence of these MMPs in gliomas, and their established roles as ECM-degrading enzymes, we speculate that these proteases mediate glioma invasion into normal tissue through degradation of the surrounding matrix and influence tumor neovascularization via the degradation of vascular basement membranes, promoting angiogenesis.

The sections studied are narrowly selected for practical reasons such as availability of high quality tissue that

Fig. 3. Paraffin sections of normal brain and glioma tissue specimens stained using in situ hybridization (A, B, C, and D) and immunohistochemistry (E, F, G, and H). A. Normal brain showing signal for mRNA in neurons, with fainter signal seen in blood vessels. B, C, and D. Glioma tissue specimens demonstrating positive signal for transcripts in cells and in vascular structures. E. No detectable protein signal observable in normal brain tissue specimens. F, G, and H. Positive immunolocalization in glioma tissue as seen cells, with faint signal in vascular structures. Original magnification X20.

Fig. 4. Paraffin sections of normal brain and glioma tissue specimens stained using in situ hybridization (A, B, C, and D) and immunohistochemistry (E, F, G, and H). A. Normal brain showing positive mRNA signal in neurons, with little signal seen in blood vessels. B, C, and D. In glioma tissue specimens, signals for transcripts as seen in tumor vasculature and glioma cells. E. No detectable signal observed for protein in normal brain. F, G, and H. In glioma tissue sections, protein immunolocalization as seen prominently in blood vessels and also in tumor cells. Original magnification X20.

included portions of invasive tumor, proliferating borders, and tumor centers. Only representative sections are presented. The expression patterns described were those most easily seen in the more cellular areas of the proliferating margins and tumor centers (unless necrosis was seen). We were unable to see reliable signals in isolated (highly invasive) tumor cells found in regions of normal brain but think our methods are too insensitive to detect very low levels of expression. In situ RT-PCR and in situ zymography of isolated glioma may characterize the expression in isolated, highly invasive glioma cells.

Matrix metalloproteinases are secreted in an inactive “pro-MMP” form and require proteolytic cleavage to become biologically active. The absence of gelatinolytic activity corresponding to activated forms in all but two samples should not be misinterpreted as suggesting there are no activated forms of these proteases present in our samples. Rather, the amounts of active MMP relative to the latent forms are too small to be detected by our zymographic technique. As well, these enzymes are under tight spatial and temporal control. For example, progelatinase-A activation by a class of membrane-bound MMPs—the membrane-type MMPs—concentrates proteolytic activity at the cell membrane. Finally, the activated forms of these proteases are subject to inhibition by their natural inhibitors, the tissue-derived

inhibitors of metalloproteinases (TIMPs). In situ zymography would help resolve these issues and allow spatial resolution of MMP activity, which is not possible with tissue homogenates.

We observed that, in contrast to systemic cancers, glioma cells, and not the surrounding stroma, express gelatinase-A and gelatinase-B. Stromal expression by systemic cancers has been interpreted as a host response by the stroma to the presence of neoplastic cells (Heppner et al., 1996). Perhaps this difference in cellular expression of MMPs in gliomas versus systemic cancers may be due to the nature of the specialized ECM stromas in the brain. The tough basement membranes and collagen-rich tissue planes that often confine systemic cancers are absent in the ECM of the brain. Instead, the ECM of the brain is very different, composed mostly of proteoglycans and hyaluronan (Giese et al., 1996). This ECM, which is less a barrier to invasive tumor cells, may render expression by the stroma less critical for glioma invasion.

We propose that gelatinase-A and gelatinase-B play a role in glioma cell invasion and that gelatinase-B has a more prominent role in angiogenesis and neovascularization of gliomas. As these are produced by both endothelial cells and glioma tumor cells, these data support the targeting of MMP inhibition as novel therapy in gliomas.

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