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Expression of a Cleaved Brain-Specific Extracellular Matrix Protein Mediates Glioma Cell Invasion *In Vivo*

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Malignant gliomas (primary brain tumors) aggressively invade the surrounding normal brain. This invasive ability is not demonstrated by brain metastases of nonglial cancers. The brain-specific, brain-enriched hyaluronan binding (BEHAB)/brevican gene, which encodes an extracellular hyaluronan-binding protein, is consistently expressed by human glioma and is not expressed by tumors of nonglial origin (Jaworski et al., 1996). BEHAB/brevican can be cleaved into an N-terminal fragment that contains a hyaluronan-binding domain (HABD) and a C-terminal fragment (Yamada et al., 1995). Here, using antisera to peptides in the predicted N-terminal and C-terminal proteolytic fragments, we demonstrate that the BEHAB/brevican protein is cleaved in invasive human and rodent gliomas. A role for this protein in glioma cell invasion was tested by transfecting a noninvasive cell line with the BEHAB/brevican gene. The noninvasive 9L glioma cell was transfected with either full-length BEHAB/brevican or the HABD and tested for invasion in *in vitro* and *in vivo* invasion assays. Although both constructs increased invasion *in vitro*, only the HABD increased invasion by tumors growing *in vivo*. Experimental intracranial tumors from full-length transfectants showed no increase in invasion over control tumors, whereas tumors from HABD transfectants showed a marked potentiation of tumor invasion, producing new tumor foci at sites distant from the main tumor mass. This work demonstrates a role for a brain-specific extracellular matrix protein in glioma invasion, opening new therapeutic avenues for a uniformly fatal disease.

Key words: glioma; brain tumor; astrocytoma; tumorogenesis; motility; proteoglycan; BEHAB; brevican; invasion; extracellular matrix

Primary tumors of the CNS (gliomas) are notoriously difficult to control, attributable in large measure to their highly invasive behavior. The ability to invade into the surrounding normal brain is essentially a property unique to primary brain tumor cells; nonglial tumor cells that metastasize to brain, such as those originating from primary tumors elsewhere (e.g., breast or lung), grow as circumscribed masses with well-defined borders. The composition of the extracellular matrix may be a critical factor in determining the invasive potential of cancer cells, such that the production of matrix elements by glioma cells might mediate their invasion into normal tissue.

The composition of the extracellular matrix of the brain changes over the course of development (Herndon and Lander, 1990; Hockfield, 1990; Sheppard et al., 1991). Behaviors characteristic of cells in the developing brain, such as cell proliferation and migration, neuronal and glial process outgrowth, and the elaboration of the capillary network, take place in a soluble matrix that is permissive for cell movement. In contrast, there is little cell motility in the normal mature brain. The matrix of the mature brain is relatively insoluble compared with that of the immature brain and can stabilize mature cell–cell relationships (Hockfield et al., 1990). A return to an immature, more soluble matrix during tumor growth could facilitate tumor cell motility and angiogenesis.

We recently cloned the gene for a brain-specific extracellular matrix protein, BEHAB (brain-enriched hyaluronan binding) (Jaworski et al., 1994), which was independently cloned in another laboratory and named brevican (Yamada et al., 1994). BEHAB/brevican encodes a member of the proteoglycan tandem-repeat family of proteins, and its predicted product is a secreted protein with a hyaluronan-binding domain (HABD) (Jaworski et al., 1994; Yamada et al., 1994). A GPI-anchored isoform of this protein has also been reported (Seidenbecher et al., 1995). The BEHAB/brevican gene is expressed at high levels during the period when glial cells, which give rise to primary brain tumors, are first born (Jaworski et al., 1995). BEHAB/brevican is also expressed in tumors derived from glial cells (HABD) (Jaworski et al., 1996). In every one of over 40 surgical samples of human glioma assayed to date, including oligodendroglioma, all grades of astrocytoma, and gliosarcoma, BEHAB/brevican mRNA is detected. By contrast, BEHAB/brevican is undetectable in tumors that are not of glial origin, including CNS lymphoma, meningioma, and carcinomas of the lung, colon, and breast, in either primary locations or as metastases to the brain. Importantly, BEHAB/brevican is expressed at very low levels in the normal adult human brain and in brain samples from individuals with noncancer neuropathologies (Jaworski et al., 1996). In rodent brain tumor models, glioma cell lines that reproduce the invasive behavior characteristic of human glioma express BEHAB/brevican mRNA, whereas glioma cell lines that grow non-

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invasively do not express it (Jaworski et al., 1996). The expression in invasive tumors, together with the predicted structure of the protein, suggests that BEHAB/brevican plays a role in glioma cell invasion.

Here, BEHAB/brevican antibodies have been used to study the expression of the protein in invasive human and rodent brain tumors. We show that in these tumors, BEHAB/brevican is cleaved into an N-terminal fragment containing the hyaluronan-binding domain (HABD) and a C-terminal fragment. To test for a role for BEHAB/brevican in tumor invasion, we transfected a noninvasive glioma cell line with either the full-length BEHAB/brevican gene or with a construct encoding the N-terminal HABD. Although both constructs increase invasion *in vitro*, in both a Matrigel invasion assay and a motility assay, only the HABD fragment increases the invasion of experimental tumors *in vivo*. These results offer an explanation for the unusual invasive ability of brain tumor cells and identify new therapeutic targets for a uniformly fatal disease.

MATERIALS AND METHODS

Cell transfections. 9L gliosarcoma cells were transfected either with a full-length cDNA encoding the secreted form of rat BEHAB/brevican (generously provided by Dr. Yu Yamaguchi, Burnham Institute) (Yamada et al., 1995) or with a 1.1 kb cDNA (nucleotides 60–1172 of the full-length clone) encoding the HABD of BEHAB/brevican by either calcium phosphate coprecipitation or electroporation. The cDNAs were cloned into the *Eco*RI site of the eukaryotic expression vector pCDNA3 (Invitrogen, San Diego, CA). Transfectants were selected in DMEM with 10% fetal bovine serum (FBS) and 1 mg/ml G418. After 10 to 14 d, G418-resistant colonies were isolated and assayed for BEHAB/brevican or HABD expression by RNase protection and Northern blot. Transfectants expressing an appropriately sized mRNA that hybridized with a BEHAB/brevican-specific probe were subcloned and used for further studies. As a control, 9L cells were transfected with the pCDNA3 vector containing a cDNA insert encoding green fluorescent protein (GFP) (generously provided by Dr. Thom Hughes, Yale University). Stable transfectants were maintained in DMEM with 10% FBS and 500 μg/ml G418. The rat CNS-1 glioma cell line was maintained in RPMI with 10% FBS.

Western blot analysis. Five milliliters of OPTI-MEM (Life Technologies) with 1% FBS were added to cultures when cells reached 80% confluence on 100 mm culture plates. After 48 hr, conditioned medium was collected, and cell debris was removed by centrifugation. For cell homogenates, cultures were rinsed in Dulbecco's PBS (DPBS; Life Technologies) with a cocktail of protease inhibitors (Boehringer Mannheim, Indianapolis, IN), and cells were collected by scraping. For tumor samples, tissues were homogenized in DPBS with protease inhibitors. Samples were electrophoresed on either 8 or 10% SDS-polyacrylamide gels, and proteins were then electrophoretically transferred to nitrocellulose. Blots were incubated with specific rabbit primary antisera (see below), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibodies (Promega, Madison, WI). Immunoreactive bands were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO).

Matrigel invasion assay. To study the invasive ability of tumor cells, we performed an *in vitro* Matrigel assay (Mohanam et al., 1993). Briefly, 100 μl of a Matrigel solution (Collaborative Research, Bedford, MA; 1 mg/ml in DMEM) was placed on a Transwell insert (Costar, Cambridge, MA; 12 mm, 8 μm pore size) and allowed to gel at 37°C for 30–40 min. Tumor cells were suspended in medium (100 cells/μl of DMEM with 10% FBS), and 100 μl aliquots were added to each Matrigel-coated Transwell insert. The lower chamber of the Transwell was filled with 500 μl of DMEM with 10% FBS to which either fibronectin (5 μg/ml) or hyaluronan (HA) (200 μg/ml) was added as a chemoattractant. Either 18 or 6 hr later, cultures were fixed in acid alcohol and stained with Coomassie blue (0.1% in 50% methanol with 7.5% acetic acid). Cells on the upper side of the insert membrane were removed with a cotton swab, and the number of cells that had migrated to the lower side of the membrane was counted. For each membrane, eight random fields were selected, and the number of cells was counted on an inverted microscope using a 20× objective lens.

Transwell motility assay. The assay was performed essentially as described for the Matrigel assay (above), with the exception that the Transwell insert was uncoated. Tumor cells (10,000 cells/well) were applied to the Transwell membrane, and the lower chamber was filled with medium (DMEM with 10% FBS, supplemented with HA at 200 μg/ml). Six hours later, cultures were processed and analyzed as described above for the Matrigel assay.

Antibodies. Rabbit antisera to BEHAB/brevican were generated to a peptide in the HABD (amino acids 253–279, DLNGELFLGAPPGKLTWEEARDYCLER) or to a peptide in the C-terminal fragment (amino acids 506–529, SPSPRPPRVHGPPAETLQPPREGS). Antisera were affinity-purified, and specific immunoreactivity was confirmed by blocking with specific peptides.

Intracranial grafts. Intracranial grafts were performed as described previously (Jaworski et al., 1996). Briefly, cell suspensions were prepared in complete PBS (PBS supplemented with 1 μg/ml $MgCl_2$ and $CaCl_2$ and 0.1% glucose) at 1–5 × 10^4 cells/μl. Using a Hamilton syringe, we injected stereotaxically 3 μl of the cell suspension over a 4–5 min period into the thalamus of a postnatal day 45 rat (Lewis for CNS-1 cells; Fischer 344 for 9L-transfected cell lines). Ten to fifteen days after the injection, the rats were killed, and the brains were quickly frozen on dry ice. Each brain was sectioned at 20 μm onto gelatin-subbed slides, and the sections were stained with cresyl violet to visualize tumor cells. Sections were also stained with an antibody to nestin (monoclonal antibody Rat-401) (Hockfield and McKay, 1985) that recognizes glioma cells. An identical distribution of tumor cells was seen in sections stained with either cresyl violet or Rat-401. Images of random sections through each tumor were captured on a computer. Using the National Institutes of Health Image program, we determined the border of the tumor with the underlying thalamus, and the number of cell clusters at distances of 0.5–1 mm and over 1 mm from the tumor border was counted in each section. The statistical analyses incorporated one random section from each of several independent tumors ($n = 6$ independent tumors for 9L-GFP; $n = 12$ for 9L-BEHAB/brevican; and $n = 14$ for 9L-HABD).

Statistical analyses. All statistical analyses were performed using the Student's t test; the level of significance was set at $p < 0.01$.

RESULTS

To begin our studies to determine whether BEHAB/brevican might play a role in tumor invasion, we assayed protein expression in rodent and human brain tumors. Experimental brain tumors established from the rat CNS-1 and 9L glioma cell lines, when implanted as intracranial grafts in syngeneic hosts, show two different patterns of growth (Jaworski et al., 1996). Intracranial grafts of the 9L gliosarcoma line display properties characteristic of brain metastases of peripheral tumors, in that they do not express BEHAB/brevican mRNA and they grow as well-defined cell masses that do not invade the surrounding brain tissue. In marked contrast, intracranial grafts of the CNS-1 glioma cell line display properties characteristic of human glioma, in that they express BEHAB/brevican mRNA and grow invasively. To study the protein product of the BEHAB/brevican mRNA, we examined BEHAB/brevican protein expression in experimental rodent brain tumors. Tumors established from the 9L cell line do not express BEHAB/brevican protein. In contrast, Western blots of brain tumors established from CNS-1 cells show three major immunoreactive bands (Fig. 1*A*). The 140 kDa band represents full-length BEHAB/brevican, and the 90 and 50 kDa bands represent C- and N-terminal cleavage products, respectively, from a predicted, conserved proteolytic site (Yamada et al., 1995). The 140 and 90 kDa forms correspond to the 140 and 80 kDa bands reported previously (Seidenbecher et al., 1995; Yamada et al., 1995). Surgical samples of human gliomas were also analyzed by Western blotting, and these, like the invasive rodent tumors, showed both full-length and cleaved protein products of the BEHAB/brevican gene (Fig. 1*B*) that were of slightly greater apparent molecular mass.

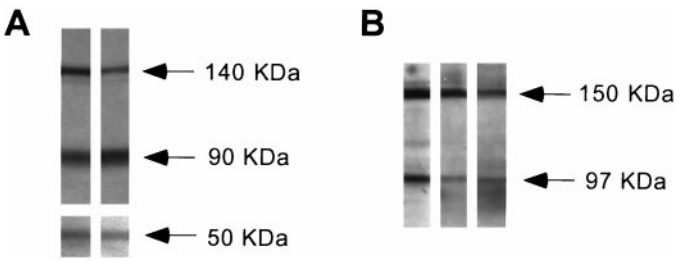

Figure 1. Expression of BEHAB/brevican in rodent and human brain tumors. *A*, Western blots of invasive rodent brain tumors established from CNS-1 cells show full-length 140 kDa BEHAB/brevican, as well as 90 and 50 kDa proteolytic fragments. Full-length and cleaved 90 kDa bands are visualized with an antibody to a peptide in the C-terminal portion of BEHAB/brevican; the 50 kDa band is visualized with an antibody to a peptide in the N-terminal portion of BEHAB/brevican (see Materials and Methods). *B*, Western blots of surgical samples from neuropathologically diagnosed glioblastoma multiforme show 150 kDa full-length BEHAB/brevican and a 97 kDa proteolytic fragment. These bands are immunoreactive with the antibody to the C-terminal peptide; however, the antibody to the N-terminal peptide does not recognize the human protein.

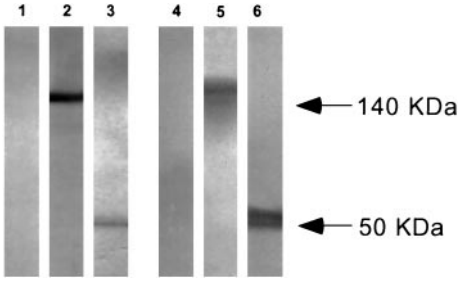

Figure 2. Transfected 9L cells express full-length BEHAB/brevican or the HABD. Western blots of cell homogenates (*lanes 1–3*) and conditioned media (*lanes 4–6*) are from transfected cells. 9L-GFP cell homogenates (*lane 1*) and conditioned media (*lane 4*) show no immunoreactivity for BEHAB/brevican or its cleavage products. 9L-BEHAB/brevican cell homogenates (*lane 2*) and conditioned media (*lane 5*) contain a 140 kDa-immunoreactive species. 9L-HABD cell homogenates (*lane 3*) and conditioned media (*lane 6*) contain a 50 kDa species. The immunoreactive species in conditioned media from both 9L-BEHAB/brevican and 9L-HABD transfectants are polydisperse (*lanes 5, 6*), possibly reflecting glycosylation.

To test whether BEHAB/brevican mediates invasion, we established stable transfectants of the noninvasive 9L gliosarcoma cell line. One set of transfectants, 9L-BEHAB/brevican, carried a construct encoding full-length BEHAB/brevican (amino acids 1–883), and a second set, 9L-HABD, carried a construct encoding the N-terminal HABD (amino acids 1–371), which corresponds approximately to the 50 kDa cleavage product. 9L cells transfected with the gene for the GFP served as a control (9L-GFP). Expression of appropriate mRNA was demonstrated by RNase protection and Northern blot analyses (data not shown). Western blot analysis confirmed the expression of the predicted proteins (Fig. 2). 9L-GFP transfectants showed no BEHAB/brevican immunoreactivity, whereas cell homogenates of 9L-BEHAB/brevican and 9L-HABD transfectants exhibited a 140 kDa- and a 50 kDa-immunoreactive species, respectively. Both 9L-BEHAB/brevican and 9L-HABD transfectants secreted the encoded proteins into the medium in which they were maintained (Fig. 2, *lanes 5, 6*). The secreted proteins were more disperse and slightly larger in apparent molecular mass, most likely because of glycosylation. Immunoreactivity on Western blots of both 9L-BEHAB/brevican and 9L-HABD transfectants was blocked by specific, but not by irrelevant, peptides (data not shown).

Transfected cells were first tested for invasive ability using the Matrigel *in vitro* invasion assay (Albini et al., 1987; Merzak et al., 1994). Eighteen hours after plating onto Matrigel-coated Transwell inserts, the number of cells that had migrated through the Matrigel to the bottom of the filter was counted (Fig. 3*A*). Two independent lines from each construct were assayed. 9L-GFP transfectants showed little migration under any condition tested. Both 9L-BEHAB/brevican and 9L-HABD transfectants showed a marked increase in invasive ability over that seen with the control 9L-GFP cells. There was no statistically significant difference between the invasive capacity of 9L-BEHAB/brevican and 9L-HABD transfectants. Invasion through Matrigel was assayed using both fibronectin and hyaluronan as attractants in the lower chamber of the Transwell apparatus, and there was no difference in the response of either cell type to the different attractants. When the Matrigel assay was performed using a 6 hr incubation period, similar results were found; 9L-BEHAB/brevican and 9L-HABD cells showed equivalent increases in invasiveness over 9L-GFP cells (data not shown).

To eliminate the possibility that the full-length BEHAB/brevican was cleaved by proteases in the Matrigel, two experiments were performed. First, heat-inactivated Matrigel (56°; 1 hr) was used for the invasion assay, which gave identical results to those described above. Second, another motility assay was performed that did not require Matrigel. We performed a standard cell motility assay using a Transwell apparatus (Koochekpour et al., 1995); cells were plated onto the Transwell inserts without any Matrigel coating. Six hours later, the number of cells that migrated to the lower side of the insert membrane was counted. In the absence of Matrigel, both 9L-BEHAB/brevican and 9L-HABD cells showed a marked increase in motility over 9L-GFP cells (Fig. 3*B*). As seen in the Matrigel invasion assay, there was not a significant difference between the motility of cells expressing either the full-length or the HABD protein.

Matrigel provides a reproducible, uniform matrix; however, its composition is quite different from that of the extracellular matrix of brain. Matrigel and the extracellular matrix of most tissues contain collagen, laminin, and fibronectin, constituents that are lacking or present at extremely low abundance in the extracellular matrix of the adult brain (Lander and Hockfield, 1998). Therefore, cell migration through Matrigel may not accurately reflect the ability of cells to invade within the matrix of the brain. To test the ability of BEHAB/brevican to mediate invasion *in situ*, we used an *in vivo* brain tumor invasion model. Transfected 9L cells were injected into the diencephalon of adult rats. Ten to 15 d after injection, the brains were sectioned to study tumor growth and the movement of tumor cells away from the main tumor mass into the underlying thalamus (Fig. 4). As in the *in vitro* assay, two independent lines for each transfectant were tested for invasion *in vivo*.

Intracranial grafts of 9L-GFP transfectants ($n = 6$) showed the same histological pattern reported previously for parental, untransfected 9L cells (Jaworski et al., 1996). That is, they grew as highly compact cell masses, with relatively smooth borders (Fig. 4*A*,*A′*). Very few clusters of 9L-GFP cells were found distal to the boundary between the tumor and the underlying thalamus (Fig. 4*A′*, Table 1). In all of the sections from 9L-GFP tumors assayed, only two cell clusters were observed over 1 mm from the tumor border. The behavior of tumors resulting from 9L-BEHAB/brevican transfectants ($n = 12$) was indistinguishable from that of tumors resulting from 9L-GFP transfectants. The

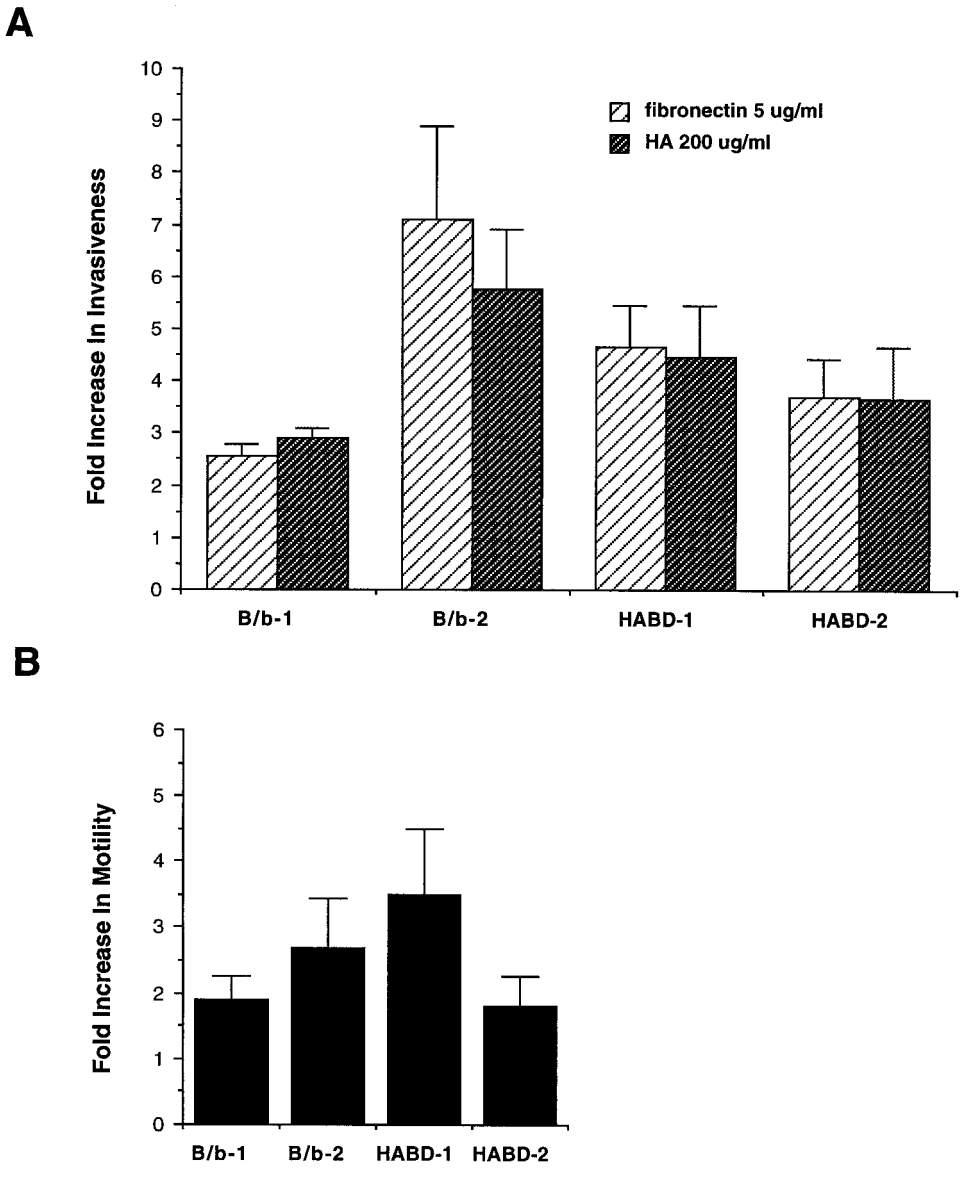

Figure 3. Full-length BEHAB/brevican and the HABD increase invasion and motility of 9L cells *in vitro*. *A*, Invasion in the Matrigel invasion assay is potentiated by the expression of either full-length BEHAB/brevican or the HABD. Two independent cell lines for each of the constructs (*B/b-1* and *B/b-2* for 9L-BEHAB/brevican; *HABD-1* and *HABD-2* for 9L-HABD) were assayed for invasion in the Matrigel invasion assay (see Materials and Methods). Invasion is expressed relative to that observed for the 9L-GFP transfectants. Both constructs markedly increased invasion. Invasion was equivalent when either fibronectin or hyaluronan was the attractant in the lower chamber. *B*, Motility in the absence of Matrigel is also increased by the expression of either full-length BEHAB/brevican or the HABD. As seen in the Matrigel assay, both constructs similarly increased motility in a Matrigel-independent assay. The degree of invasion or motility seen for all four transfected cell lines was statistically different from 9L-GFP cells at the $p < 0.01$ level. There was not a statistically significant difference between the behavior of 9L-BEHAB/brevican versus 9L-HABD transfectants. Error bar indicates SEM.

9L-BEHAB/brevican tumors also grew as compact cell masses, with few cell clusters located outside of the main tumor mass (Fig. 4*B*,*B′*). Of the few clusters of cells that were observed beyond the border of the tumor with the underlying thalamus, almost all were located 0.5–1 mm from the tumor border (Table 1). Western blots of 9L-BEHAB/brevican tumors detected only the 140 kDa full-length protein (Fig. 5, *lane 1*). Therefore, although the expression of full-length BEHAB/brevican can increase the migration of 9L cells in an *in vitro* invasion assay, when tested *in vivo*, expression of the full-length protein does not confer on 9L cells the ability to migrate through normal adult brain matrix.

The lack of invasion by 9L-BEHAB/brevican transfectants *in vivo*, along with the demonstration of proteolytic cleavage products in human gliomas and invasive rat brain tumors, led us to test whether the HABD of BEHAB/brevican might mediate invasion *in vivo*. The behavior of 9L-HABD transfectants ($n = 14$) was markedly different from that of 9L-GFP and 9L-BEHAB/brevican transfectants (Fig. 4*C*,*C′*). In 9L-HABD experimental tumors, the border between the tumor and the underlying thalamus was interrupted by peninsulas of cells extending from the main tumor mass (Fig. 4*C′*). In addition, there were numerous cell clusters 0.5–1.0 mm from the tumor border, with many cell clusters located over 1 mm from the main tumor mass (Table 1). Western blots of 9L-HABD tumor samples showed the presence of the 50–55 kDa HABD product (Fig. 5, *lane 2*). Therefore, expression of the HABD of BEHAB/brevican can increase the ability of 9L cells to migrate through the brain matrix *in vivo*.

DISCUSSION

We show here that, within the brain, invasive ability can be conferred on a noninvasive glioma cell line by expression of an N-terminal fragment of the BEHAB/brevican protein but not by expression of the full-length protein. Because partial proteolytic cleavage of the BEHAB/brevican protein occurs endogenously in both human and rodent invasive brain tumors, these results provide an explanation for glioma cell motility in the adult human brain. In addition, they suggest new therapeutic possibilities for human brain tumor.

Many studies have suggested a central role for extracellular matrix proteins in tumor growth and motility (Paulus et al., 1996;

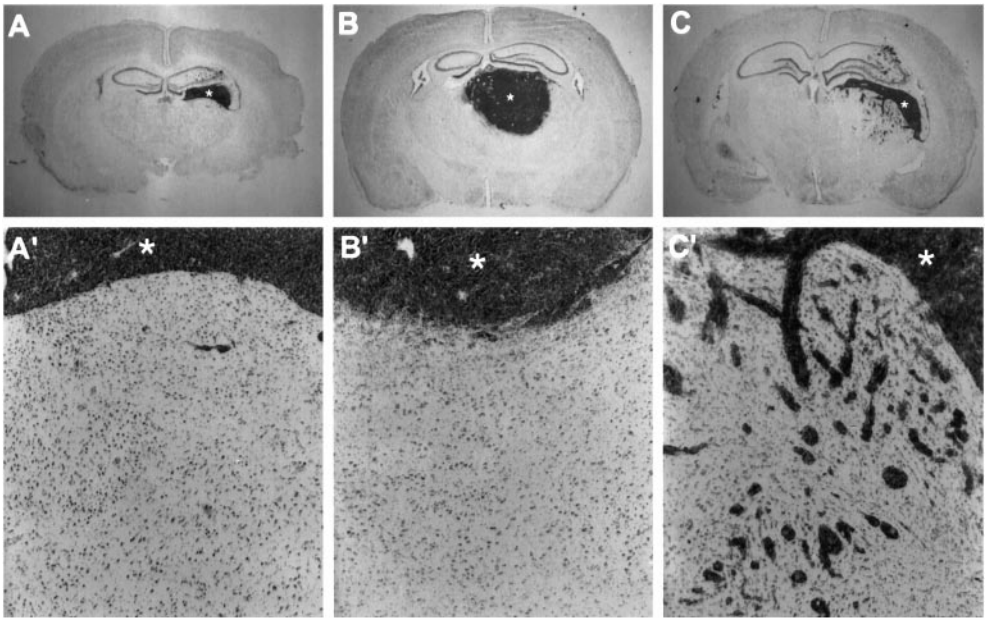

Figure 4. Intracranial tumors established from 9L-HABD, but not from 9L-BEHAB/brevican, cells show increased invasion into the surrounding brain. *A*, *A′*, Intracranial tumors (*asterisks*) derived from 9L-GFP cells grow as compact cell masses, with little infiltration into the surrounding brain. The border with the underlying thalamus (*A′*) is smooth, with very few clusters of cells seen beyond the border between the tumor and the normal brain. *B*, *B′*, Intracranial tumors (*asterisks*) derived from 9L-BEHAB/brevican cells showed identical behaviors to those of the control transfectants. These tumors also grew as compact cell masses, with little infiltration of the surrounding brain. Here again, very few cell clusters were observed in the normal brain adjacent to the tumor (*B′*). *C*, *C′*, Intracranial tumors (*asterisks*) derived from 9L-HABD cells showed a marked increase in invasive ability compared with the other two cell lines. Although the main tumor mass was a compact-group of cells, many cell clusters were seen in the surrounding normal brain. The border of the 9L-HABD tumors with the underlying thalamus (*C′*) was often interrupted by peninsulas of cells extending out from the main tumor mass.

Table 1. Number and distance of tumor cell clusters from the main tumor mass

	0.5–1.0 mm	>1.0 mm
9L-GFP	1.67 ± 0.76	0.33 ± 0.33
9L-BEHAB/brevican	1.69 ± 0.77	0.15 ± 0.10
9L-HABD	8.33 ± 2.02	8.75 ± 3.70

The number of cell clusters located 0.5–1 mm and over 1 mm from the main tumor mass was counted for all of the tumors in this experiment (one random section per tumor; $n = 6$ independent tumors for 9L-GFP; $n = 12$ for 9L-BEHAB/brevican; and $n = 14$ for 9L-HABD). Two different transfected lines were used for each of the noncontrol transfectants. The HABD fragment markedly increased the number of cell clusters seen distant to the main tumor mass. The full-length BEHAB/brevican protein had no effect on cell infiltration into surrounding brain over that observed for the control 9L-GFP construct. The difference between the 9L-HABD versus the control 9L-GFP and 9L-BEHAB/brevican transfectants was statistically different at the $p < 0.01$ level. Data shown here are the grouped averages of the number of cell clusters ± SEM.

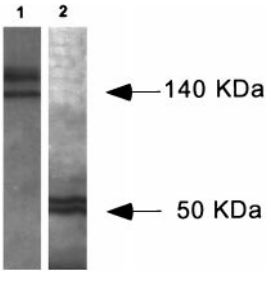

Figure 5. Intracranial tumors established from 9L-BEHAB/brevican and 9L-HABD cells express full-length or HABD proteins. Western blots of tumors produced after intracranial injection of 9L-BEHAB/brevican (*lane 1*) or HABD (*lane 2*) transfectants are shown. In 9L-BEHAB/brevican tumors (*lane 1*), immunoreactivity for BEHAB/brevican is at 140 kDa. No evidence of a cleavage product is seen. In 9L-HABD tumors (*lane 2*), only a 50 kDa product is observed.

Haugland et al., 1997; Merzak and Pilkington, 1997). HA and HA-binding proteins have been implicated in tumor metastasis (Knudson and Knudson, 1993; Entwistle et al., 1996). For example, the HA-binding protein RHAMM increases the metastatic potential of fibroblast cell lines (Hall et al., 1995). Our present results provide evidence that expression of the extracellular, HA-binding, BEHAB/brevican protein by invasive rodent and human brain tumors can increase the ability of glioma cells to invade into the surrounding normal brain.

Although both 9L-BEHAB/brevican and 9L-HABD cells showed an equivalent increase in invasive ability in the *in vitro* Matrigel invasion assay compared with control cells, only the 9L-HABD cells showed an increase in invasion when grown as intracranial grafts. Therefore, an increase in invasive ability observed in the Matrigel assay did not necessarily predict an increase in invasive ability in the brain. One explanation for the invasive behavior of 9L-BEHAB/brevican cells in Matrigel, but not in the brain, is that glioma invasion requires proteolysis of the BEHAB/brevican protein, and the Matrigel might contain the necessary proteases. Our results indicate that proteolysis is not likely to be responsible for the migration of 9L-BEHAB/brevican cells through Matrigel. First, heat inactivation of the Matrigel, which should inactivate protease activity, did not alter the invasive ability of 9L-BEHAB/brevican transfectants. Second, 9L-BEHAB/brevican cells also migrated in a Matrigel-free motility assay. Therefore, it is unlikely that cleavage of the protein explains the motility of the 9L-BEHAB/brevican transfectants in the *in vitro* assays. A more likely explanation is that, whereas the full-length BEHAB/brevican protein can increase invasion and motility on extracellular matrix elements of non-neural origin, it does not increase invasion or motility on the extracellular matrix of the brain. This result suggests that caution be exercised in the interpretation of results from *in vitro* assays, because the behavior of cells in *in vitro* assays may not accurately predict the behavior of cells *in vivo*. A similar discrepancy has been reported for other tumor cell types, in which increased invasiveness in a Matrigel assay was not paralleled by increased invasiveness *in vivo* (Noel et

al., 1991). Invasion through normal brain tissue may be particularly difficult to model *in vitro* because of the unusual and heterogeneous composition of the extracellular matrix of the brain (Lander et al., 1997).

In the normal adult human brain, the level of BEHAB/brevican expression is extremely low. In the normal adult rat brain, however, BEHAB/brevican is expressed at easily detectable levels (Jaworski et al., 1994; Yamada et al., 1994; Yamaguchi, 1996). It is important to note that, although cells in the rat brain surrounding an invasive tumor express BEHAB/brevican, the level of expression by invasive tumors (such as those derived from either C6 or CNS-1 glioma cells) is markedly higher than that in the surrounding normal brain (Jaworski et al., 1996). Therefore, the level of cleaved BEHAB/brevican in the surrounding brain may not be sufficient to permit invasion by the 9L-GFP or full-length 9L-BEHAB/brevican transfectants. Alternatively, invasion may require expression of BEHAB/brevican by the tumor cells themselves, perhaps involving an interaction of the cleavage product(s) with the glioma cell surface (Yamada et al., 1997). Although expression of an N-terminal HABD fragment of BEHAB/brevican clearly increases the invasive ability of 9L cells, the pattern of invasion of 9L-HABD transfectants does not fully mimic that of the highly invasive CNS-1 and C6 cell lines (Jaworski et al., 1996), suggesting that additional molecules may act to facilitate glioma invasion further.

The BEHAB/brevican gene is expressed as two isoforms, one secreted and one GPI-linked (Seidenbecher et al., 1995). In the normal rat brain (Seidenbecher et al., 1995) and in human brain tumors (J. Gaw, V. Chang, and S. Hockfield, unpublished observations), the GPI-linked form is expressed at very low levels compared with the secreted form. The role, if any, of the GPI-linked form in tumor invasion is currently under investigation. The most important modification of the BEHAB/brevican protein product for cell invasion appears to be cleavage into the N-terminal fragment containing an HABD and the C-terminal fragment. A predicted site for proteolytic cleavage of BEHAB/brevican has been mapped and is highly conserved with a proteolytic site of another ECM protein, aggrecan (Yamada et al., 1995; Yamaguchi, 1996). The observations that BEHAB/brevican is cleaved in invasive human and rodent tumors and that full-length BEHAB/brevican does not mediate invasion *in vivo* together indicate that proteolytic cleavage is required for invasion. Moreover, cleavage of the protein appears to require an association of the protease activity with the cell that expresses the protein, because the protein produced by 9L-full-length transfectants remains uncleaved (Fig. 5, *lane 1*), whereas the endogenous BEHAB/brevican expressed in the surrounding brain is cleaved.

The results presented here are consistent with a two-component mechanism for brain tumor cell invasion: first, an upregulation in the expression of BEHAB/brevican by glioma cells, followed by the cleavage of the protein into N-terminal HABD and C-terminal fragments. Earlier work from our laboratory indicates that expression of BEHAB/brevican by glioma cells is regulated by a brain-derived factor (Jaworski et al., 1996). When maintained under standard cell culture conditions or when grown as subcutaneous grafts outside of the brain, none of the 19 glioma cell lines we have tested expresses BEHAB/brevican. When glioma cell lines are grown as intracranial grafts, however, cell lines that grow with an invasive phenotype characteristic of human glioma are induced to express BEHAB/brevican. Cell lines that grow as noninvasive intracranial tumors do not express the gene. The identity of the BEHAB/brevican-inducing factor has yet to be determined but appears to be a soluble, brain-specific factor. In addition, the results presented here provide evidence of a second required component, a protease that cleaves BEHAB/brevican, the identity of which we are currently pursuing. Candidate proteases include the matrix metalloproteinases expressed in gliomas (Merzak and Pilkington, 1997), for which BEHAB/brevican is a possible substrate. Regulated cleavage of extracellular matrix proteins may be a general mechanism for the control of cell motility; recent studies have demonstrated that cleavage of laminin-5 is required for breast epithelial cell migration (Giannelli et al., 1997) and that inhibition of matrix metalloproteinase activity during embryogenesis blocks myoblast migration into the tongue (Chin and Werb, 1997).

The aggressive invasion by malignant glioma into the surrounding normal brain makes brain tumors highly refractory to regional therapies, such as surgery or focal irradiation. BEHAB/brevican is expressed with unprecedented specificity in glioma and presents a novel potential target for the treatment of malignant primary brain tumor. Here, we have shown that invasion by glioma cells into normal brain is potentiated by a cleavage product of BEHAB/brevican. In addition to targeting the BEHAB/brevican gene or its products, the present work suggests another therapeutic target, the protease responsible for the cleavage of BEHAB/brevican. BEHAB/brevican is brain-specific and is expressed at almost undetectable levels in the normal adult human brain; targeted disruption of either the expression or the proteolytic cleavage of BEHAB/brevican would be predicted to have minimal deleterious consequences on normal brain tissue. Functional inhibition of BEHAB/brevican might slow the migration of tumor cells from the original tumor site(s), thereby increasing the efficacy of regional therapies.

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