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Advances in Brief

Brain Enriched Hyaluronan Binding (BEHAB)/Brevican Increases Aggressiveness of CNS-1 Gliomas in Lewis Rats[1]

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Abstract

Gliomas are the most common primary intracranial tumors. One extracellular matrix component that has been implicated in glial tumor biology is brain enriched hyaluronan binding (BEHAB)/brevican. In this study, the CNS-1 rat glioma cell line was transfected with a vector containing either a full-length BEHAB/brevican cDNA, a 5′ insert encoding the NH_2-terminal BEHAB/brevican cleavage product, or a 3′ insert encoding the COOH-terminal cleavage product. As a control, CNS-1 cells were transfected with green fluorescent protein. Rats with intracranial grafts of BEHAB/brevican-transfected CNS-1 cells displayed significantly shorter survival times than did rats with CNS-green fluorescent protein intracranial grafts ($P < 0.001$). Histological examination showed that the BEHAB/brevican-transfected tumors were just as, if not more, aggressive than control tumors, even though the BEHAB/brevican tumors had been growing for only approximately two-thirds the time as long as control tumors. These data suggest that up-regulation and proteolytic cleavage of BEHAB/brevican increase significantly the aggressiveness of glial tumors. It will be important to investigate the effect of inhibiting cleavage of BEHAB/brevican in these cells and to determine the therapeutic potential of inhibiting BEHAB/brevican cleavage in gliomas.

Introduction

Gliomas are the most common primary intracranial tumors. Their distinct ability to invade the normal surrounding tissue makes them difficult to control and nearly impossible to completely remove surgically, thus accounting for the extraordinarily high lethality associated with malignant glial tumors. The ability of tumor cells to interact with components of the surrounding ECM[4] affects numerous cellular processes, and inappropriate expression of these matrix components has been associated with glial tumor invasion, growth, and angiogenesis. One ECM component that has been implicated in glial tumor biology is BEHAB/brevican, a HA-binding member of the lectican family of chondroitin sulfate proteoglycans.

BEHAB/brevican is up-regulated in the vast majority of surgical samples of glioma analyzed to date (1–3), and a role in glial tumor invasion has been suggested (1, 2, 4, 5). Recently, Zhang *et al.* (4) demonstrated that when the noninvasive 9L gliosarcoma cell line is transfected with a transgene encoding an NH_2-terminal fragment (the HA-binding domain) of BEHAB/brevican, tumors from these transfected cells display a potentiation of tumor invasion, producing new tumor foci at sites distant from the main tumor mass. In contrast, intracranial tumors of 9L cells transfected with full-length BEHAB/brevican do not grow invasively into the surrounding normal brain (4). Subsequently, it was shown that 9L cells are not capable of cleaving the full-length BEHAB/brevican protein, whereas NH_2-terminal and COOH-terminal cleavage products have been demonstrated in human gliomas (6). Taken together, these studies suggest a two-step mechanism whereby both up-regulation and proteolytic cleavage of BEHAB/brevican are critical elements for glial tumor invasion. In this study, we have used the rat glioma cell line, CNS-1. Similar to human gliomas, intracranial tumors grown from these cells are invasive, express BEHAB/brevican, and are capable of cleaving the full-length protein. Therefore, CNS-1 cells provide a model system that closely mimics the behavior of human gliomas and permits an investigation of the effects of BEHAB/brevican up-regulation in glioma. This study investigates, for the first time, the effect of BEHAB/brevican up-regulation on animal survival and further explores a role for BEHAB/brevican in glial tumor biology.

Materials and Methods

Cell Transfections. CNS-1 rat glioma cells were transfected with a pCDNA3 vector containing the desired BEHAB/brevican insert as described previously (4). The full-length BEHAB/brevican insert (7) encoded a 2.9-kb fragment (nucleotides 1–2863); the 5′ insert encoded a 1.2-kb fragment (nucleotides 110–1294 of the full-length cDNA); and the 3′ insert encoded a 1.5-kb fragment (nucleotides 1295–2809 of the full-length cDNA). As a control, CNS-1 cells were transfected with the pCDNA3 vector containing a cDNA insert encoding GFP. Stable transfectant pools were selected in 1 mg/ml G418. Expression of the desired transgene was confirmed by Northern blot analysis, and the presence of protein was confirmed by Western blot analysis as described previously (4). All CNS-1 transfected cell lines were maintained at 5% CO_2 in RPMI 1640 supplemented with 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 400 μg/ml G418.

***In Vitro* Cell Proliferation.** *In vitro* cell proliferation was determined using the MTT assay. Cells were plated in quadruplicate at a density of 2000 cells/well in 96-well plates, and the assay was performed daily for 9 days. Briefly, 25 μl of MTT solution (2 mg/ml in PBS) were added to each well, and the plates were incubated for 2 h at 37°C. To solubilize formazan crystals, 0.04 M hydrochloric acid in isopropanol was added and mixed thoroughly. After a 1-h incubation at 37°C, cell number was quantified by measuring light absorbance (595 nm) in a Bio-Rad automated microplate reader.

Intracranial Grafts. Intracranial grafts were performed as described previously (1). Briefly, cells were harvested when ~80% confluent, washed, and resuspended in injection buffer (PBS supplemented with 1 μg/ml $MgCl_2$ and $CaCl_2$ and 0.1% glucose) at a concentration of 5×10^4 cells/μl. Cell suspension (3 μl) was injected stereotaxically into the thalamus of 45-day-old female Lewis rats over a 5-min period. Tumors were harvested either upon the death of the animal or upon evidence of compromised neurological function in the animal. Brains were quick frozen on dry ice and sectioned at 25 μm. Every tenth section was mounted onto gelatin-subbed slides and stained with cresyl violet.

Statistical Methods. *In vitro* proliferation curves were compared using the Student's *t* test. Survival curves were generated according to the method of Kaplan and Meier, and significance was determined using the log-rank test.

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[4] The abbreviations used are: ECM, extracellular matrix; BEHAB, brain enriched hyaluronan binding; CNS, central nervous system; GFP, green fluorescent protein; HA, hyaluronan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Results

***In Vitro* Proliferation of Transfected CNS-1 Cells.** To examine the effect of BEHAB/brevican up-regulation on the growth of CNS-1 cells *in vitro*, proliferation rates were determined for each transfected cell line using the MTT assay. There was no significant difference in cell doubling times among these cell lines *in vitro* (Fig. 1).

Intracranial Tumor Survival Curves. Next, the effect of BEHAB/brevican up-regulation on survival was investigated. Control- or BEHAB/brevican-transfected CNS-1 cells were injected into Lewis rats, and survival was monitored until all animals had succumbed to disease. Expression of BEHAB/brevican was confirmed at the time of cell injection by Northern and Western analysis (data not shown). Rats that received injection intracranially with BEHAB/brevican-transfected CNS-1 cells displayed significantly shorter survival times than did rats with CNS-GFP intracranial tumors ($P < 0.001$; Fig. 2). Survival times for rats that received injections with the control CNS-GFP cells are comparable with those found previously by Kruse *et al.* (8) for the CNS-1 parental cell line; they reported a survival time of 30.2 ± 5.5 days for an injection of 10^5 cells compared with our survival time of 31.5 ± 3.78 days for an injection of 1.5×10^5 cells.

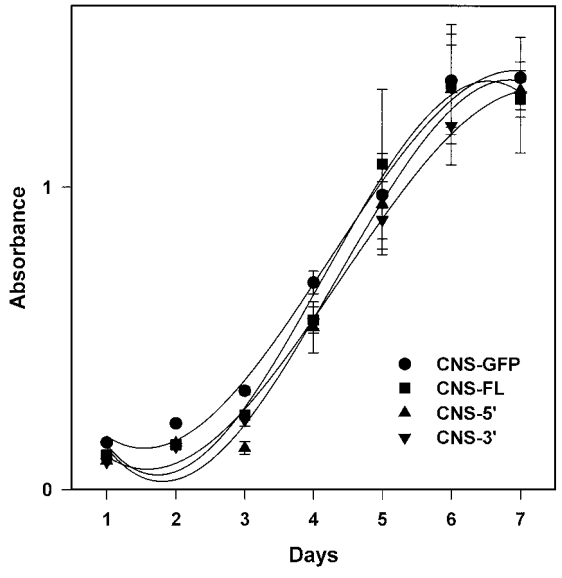


Fig. 1. *In vitro* proliferation of transfected CNS-1 cells. Growth curves were generated for each of the stably transfected pools over a 9-day period. Results for the first 7 days are shown. There was no significant difference in cell doubling times among these cell lines *in vitro*. Data are presented as the mean of four values. *Bars*, SD.

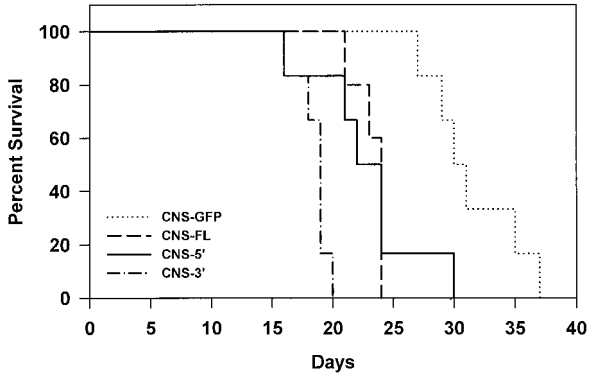
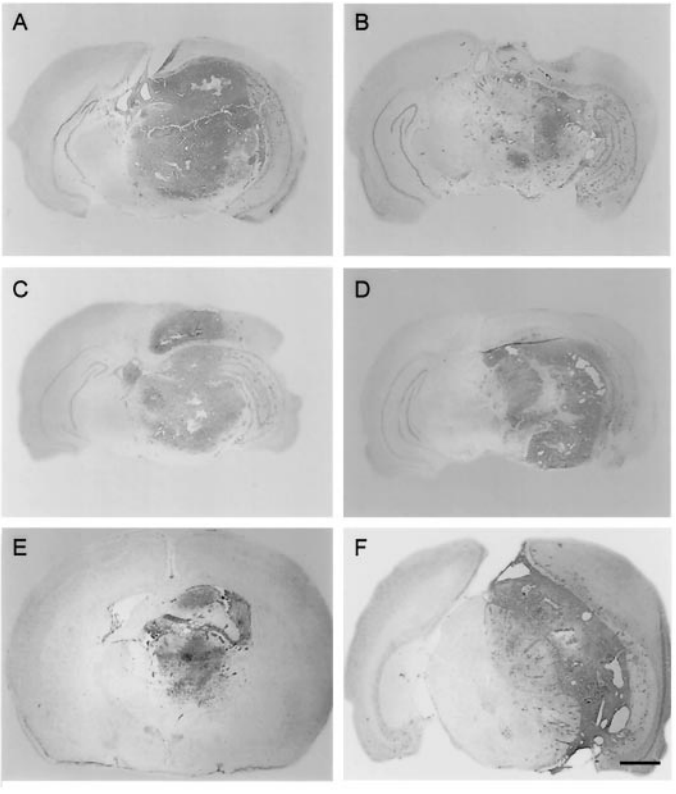


Fig. 2. Intracranial tumor survival curves. Rats that received injections intracranially with BEHAB/brevican-transfected CNS-1 cells displayed significantly shorter survival times than did rats with CNS-GFP intracranial tumors ($P < 0.001$). For each cell line, five (FL) or six (GFP, 5′, 3′) animals received injections.



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Cell Line	Average A-P Spread (mm)
CNS-GFP	9.5 ± 1.34
CNS-FL	9.8 ± 2.46
CNS-5'	9.5 ± 1.83
CNS-3'	9.95 ± 1.88

Fig. 3. Invasive behavior of transfected CNS-1 cells *in vivo*. Histological examination showed that BEHAB/brevican-transfected tumors were just as, if not more, invasive than control tumors, even though the BEHAB/brevican tumors had been growing for only approximately two-thirds of the time as control tumors. *A*, CNS-GFP-D; 31 days of survival. *B*, CNS-FL-A; 21 days of survival. *C*, CNS-5′-D; 24 days of survival. *D*, CNS-3′-B; 18 days of survival. *E*, CNS-GFP; harvested at 12 days. *F*, CNS-FL; harvested at 12 days. *G*, distance of average anterior-posterior invasion. Sections were stained with cresyl violet. *Bar*, 2 mm.

Strikingly, our average survival times of 18.5–23.2 days for the BEHAB/brevican-transfected cells are more comparable with the survival times Kruse *et al.* (8) reported for an injection of 10^6 parental CNS-1 cells (20.5 ± 3.4 days), more than seven times the number of cells we injected.

Histological Examination of Glial Tumors. From the 23 animals in the survival study, 20 tumors were available for histological examination. Of these, 4 CNS-GFP tumors were examined, 5 each of CNS-FL and CNS-3′ tumors, and 6 CNS-5′ tumors. The extent of invasion was investigated first. As can be seen in Fig. 3, *A–D*, the lateral spread of these tumors inundated the normal brain on the side of the injection site. The tumor burden of the ipsilateral tissue caused severe distortion of the structural integrity of the contralateral hemisphere; only one tumor (CNS-GFP-A; 27 days of survival) showed considerable evidence of tumor infiltrating to the contralateral side. Although the tumor burden around the injection site of these animals made it difficult to obtain an accurate assessment of the extent of lateral invasion, it does appear that the BEHAB/brevican-transfected CNS-1 cells are more invasively aggressive, as can be seen when

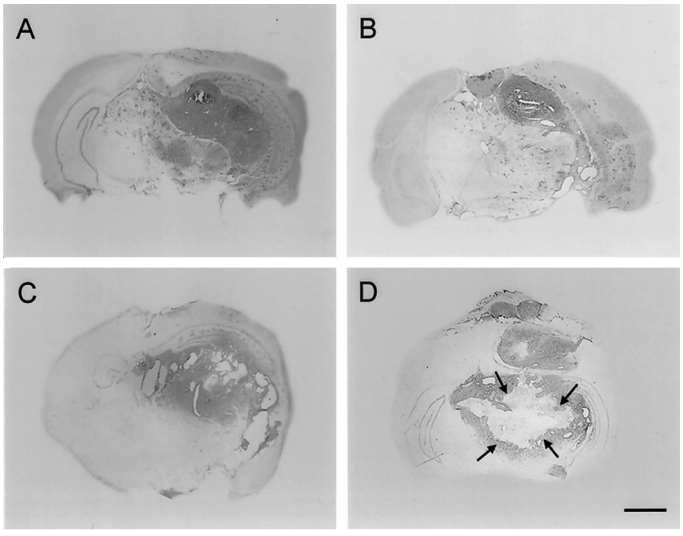

Fig. 4. Angiogenic behavior of transfected CNS-1 cells *in vivo*. BEHAB/brevican-transfected tumors appeared to be more vascular than CNS-GFP controls, and hemorrhagic regions were more evident in BEHAB/brevican-transfected tumors than controls; large hemorrhagic regions were especially prominent in CNS-3′ tumors. *A*, CNS-GFP-A; 27 days of survival. *B*, CNS-FL-A; 21 days of survival. *C*, CNS-3′-D; 19 days of survival. *D*, CNS-3′-F; 20 days of survival. Sections were stained with cresyl violet. *Arrows* in *D* demarcate the limits of a large hemorrhagic region in a CNS-3′ tumor. *Bar*, 2 mm.

tumors are harvested at an earlier time point (Fig. 3, *E* and *F*). The limits of anterior-posterior spread of these tumors also suggest that the BEHAB/brevican-transfected cells are more invasively aggressive. When the extent of anterior-posterior spread was measured, the tumor spread for the BEHAB/brevican-transfected cells appeared to be equal to or greater than the spread of the CNS-GFP cells, even though the average time of tumor incubation was much shorter (Fig. 3*G*).

In addition to invasion, the rate of growth of the tumors appeared to differ. Again, even though the average time of incubation was shorter, the BEHAB/brevican-transfected tumors appeared to be at least, if not more, cellular than the CNS-GFP control tumors. This can also be seen in the tumors that were harvested at an earlier time point (Fig. 3, *E* and *F*). Lastly, the BEHAB/brevican-transfected tumors appeared to be more vascular than the CNS-GFP controls (Fig. 4). Further, hemorrhagic regions were more evident in the BEHAB/brevican-transfected tumors than the controls; small regions of pooled blood were rarely found in CNS-GFP tumors whereas these regions were evident in all the BEHAB/brevican-transfected tumors, and large hemorrhagic regions were especially prominent in the CNS-3′ tumors (Fig. 4*D*).

Discussion

In this study, we set out to investigate the effect of BEHAB/brevican up-regulation on survival and to explore further a role for BEHAB/brevican in glial tumor biology. We demonstrated, for the first time, that rats that received injections intracranially with BEHAB/brevican-transfected CNS-1 cells displayed significantly shorter survival times than did rats with CNS-GFP intracranial tumors ($P < 0.001$). Although the BEHAB/brevican-transfected tumors had been growing for approximately two-thirds of the time as control tumors, they appeared to be just as, if not more, histologically aggressive. These data suggest that up-regulation and proteolytic cleavage of BEHAB/brevican increase significantly the aggressiveness of glial tumors. In addition, there was no evidence of increased proliferation of BEHAB/brevican-transfected cells *in vitro*, suggesting that tumor environment plays a critical role in the increased aggressiveness of glioma seen in this study.

There are several mechanisms by which BEHAB/brevican might promote glial tumorigenesis. We have proposed previously an "ECM disruption" model to explain the mechanism by which BEHAB/brevican might participate in glioma invasion (5). In addition to binding HA, BEHAB/brevican also binds the ECM protein tenascin-R (9) and a subset of sulfated cell surface glycolipids (10). BEHAB/brevican may, therefore, act as a molecular bridge between the ECM and the cell surface (11). This molecular bridge, if destabilized by cleavage in the central domain of BEHAB/brevican, could lead to a disruption of the ECM surrounding the tumor cell. In this model, coincident overexpression and cleavage of BEHAB/brevican would lead to an environment more conducive to invasion. In addition, exogenous overexpression of a cleavage product would also be expected to disrupt the integrity of the ECM through competition with full-length BEHAB/brevican. This model could also explain the more general histological effects we demonstrated in this study; a less restrictive ECM could conceivably facilitate endothelial as well as glial cell movement and, in addition, would provide an environment conducive to tumor expansion.

This model, in which glial tumorigenesis can be facilitated by alterating the restrictiveness of the brain ECM, is supported by studies that have investigated a role for HA in tumorigenesis. Kosaki *et al.* (12) demonstrated that overproduction of HA in a human fibrosarcoma cell line promoted anchorage-independent growth and tumorigenesis. An increased growth rate of these cells was not seen in monolayers, suggesting that HA required a three-dimensional environment to exert its growth-promoting effects. Furthermore, Novak *et al.* (13) demonstrated that overexpression of hyaluronidase-2 in the mouse astrocytoma cell line, SMA560, dramatically accelerated intracerebral tumor growth, and tumors from hyaluronidase-2-transfected cells were highly vascularized and more invasive than control tumors. Again, all cell lines in the Novak study demonstrated equal doubling times *in vitro* (13). It is intriguing to envision this "ECM disruption" model for BEHAB/brevican whereby a complex of BEHAB/brevican, HA, and other ECM molecules such as tenascin-R or other, as yet unidentified, proteins may form the foundation of brain ECM and that disruption of this matrix could lead to an environment conducive to increased aggressiveness of glial tumors. In addition, liberation of these molecules from the restrictive ECM may allow them to play a more direct role in tumorigenesis; for example, HA fragments can be angiogenic (14, 15).

In summary, we have demonstrated that up-regulation of BEHAB/brevican in glial tumors significantly decreases survival, and we provide evidence in support of a two-step model in which up-regulation and cleavage of BEHAB/brevican can increase the aggressiveness of glial tumors. It will be intriguing to investigate the effect of inhibiting cleavage of BEHAB/brevican in these cells; experiments such as these may provide a new therapeutic strategy for the treatment of glioma.

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References

1. Jaworski, D. M., Kelly, G. M., Piepmeier, J. M., and Hockfield, S. BEHAB (brain enriched hyaluronan binding) is expressed in surgical samples of glioma and in intracranial grafts of invasive glioma cell lines. Cancer Res., *56:* 2293–2298, 1996.
2. Gary, S. C., Kelly, G. M., and Hockfield, S. BEHAB/brevican: a brain-specific lectican implicated in gliomas and glial cell motility. Curr. Opin. Neurobiol., *8:* 576–581, 1998.

3. Gary, S. C., Zerillo, C. A., Chiang, V. L., Gaw, J. U., Gray, G., and Hockfield, S. cDNA cloning, chromosomal localization, and expression analysis of human BEHAB/brevican, a brain specific proteoglycan regulated during cortical development and in glioma. Gene (Amst.), *256:* 139–147, 2000.
4. Zhang, H., Kelly, G., Zerillo, C., Jaworski, D. M., and Hockfield, S. Expression of a cleaved brain-specific extracellular matrix protein mediates glioma cell invasion *in vivo*. J. Neurosci., *18:* 2370–2376, 1998.
5. Nutt, C. L., Matthews, R. T., and Hockfield, S. Glial tumor invasion: a role for the upregulation and cleavage of BEHAB/brevican. Neuroscientist, *7:* 113–122, 2001.
6. Matthews, R. T., Gary, S. C., Zerillo, C., Pratta, M., Solomon, K., Arner, E. C., and Hockfield, S. BEHAB/brevican cleavage in a glioma cell line is mediated by an ADAMTS family member. J. Biol. Chem., *275:* 22695–22703, 2000.
7. Yamada, H., Watanabe, K., Shimonaka, M., Yamasaki, M., and Yamaguchi, Y. cDNA cloning and the identification of an aggrecanase-like cleavage site in rat brevican. Biochem. Biophys. Res. Commun., *216:* 957–963, 1995.
8. Kruse, C. A., Molleston, M. C., Parks, E. P., Schiltz, P. M., Kleinschmidt-DeMasters, B. K., and Hickey, W. F. A rat glioma model, CNS-1, with invasive characteristics similar to those of human gliomas: a comparison to 9L gliosarcoma. J. Neuro-Oncol., *22:* 191–200, 1994.
9. Aspberg, A., Miura, R., Bourdoulous, S., Shimonaka, M., Heinegard, D., Schachner, M., Ruoslahti, E., and Yamaguchi, Y. The C-type lectin domains of lecticans, a family of aggregating chondroitin sulfate proteoglycans, bind tenascin-R by protein-protein interactions independent of carbohydrate moiety. Proc. Natl. Acad. Sci. USA, *94:* 10116–10121, 1997.
10. Miura, R., Aspberg, A., Ethell, I. M., Hagihara, K., Schnaar, R. L., Ruoslahti, E., and Yamaguchi, Y. The proteoglycan lectin domain binds sulfated cell surface glycolipids and promotes cell adhesion. J. Biol. Chem., *274:* 11431–11438, 1999.
11. Yamaguchi, Y. Lecticans: organizers of the brain extracellular matrix. Cell. Mol. Life Sci., *57:* 276–289, 2000.
12. Kosaki, R., Watanabe, K., and Yamaguchi, Y. Overproduction of hyaluronan by expression of the hyaluronan synthase Has2 enhances anchorage-independent growth and tumorigenicity. Cancer Res., *59:* 1141–1145, 1999.
13. Novak, U., Stylli, S. S., Kaye, A. H., and Lepperdinger, G. Hyaluronidase-2 overexpression accelerates intracerebral but not subcutaneous tumor formation of murine astrocytoma cells. Cancer Res., *59:* 6246–6250, 1999.
14. West, D. C., Hampson, I. N., Arnold, F., and Kumar, S. Angiogenesis induced by degradation products of hyaluronic acid. Science (Wash. DC), *228:* 1324–1326, 1985.
15. Rooney, P., Kumar, S., Ponting, J., and Wang, M. The role of hyaluronan in tumor neovascularization. Int. J. Cancer, *60:* 632–636, 1995.