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Short Communication

Perturbation of Hyaluronan Interactions Inhibits Malignant Properties of Glioma Cells

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Malignant progression of gliomas is characterized by acquisition of inappropriate growth and invasive properties. *In vitro*, these malignant properties are reflected in, and measured by, the ability to grow in an anchorage-independent manner and to invade artificial extracellular matrices. The results of numerous studies have suggested that the extracellular and pericellular matrix polysaccharide, hyaluronan, plays an important role in these attributes of malignant cancer cells. However, with respect to glioma cells, most studies have addressed the effect of exogenously added hyaluronan rather than the function of endogenous tumor cell-associated hyaluronan. In this study we manipulate hyaluronan-glioma cell interactions by two methods. The first is administration of small hyaluronan oligosaccharides that compete for endogenous hyaluronan polymer interactions, resulting in attenuation of hyaluronan-induced signaling. The second is overexpression of soluble hyaluronan-binding proteins that act as a competitive sink for interaction with endogenous hyaluronan, again leading to attenuated signaling. We find that both treatments inhibit anchorage-independent growth, as measured by colony formation in soft agar, and invasiveness, as measured by penetration of reconstituted basement membrane matrices. Based on our findings, we conclude that endogenous hyaluronan interactions are essential for these two fundamental malignant properties of glioma cells. *(Am J Pathol 2003, 162:1403–1409)*

Malignant forms of glioblastoma rarely metastasize to distant sites but they are highly proliferative and invasive tumors that destroy normal brain structure and result in high morbidity. As with many types of cancers, up-regulation of growth factor pathways and loss of tumor suppressors have been implicated in glioma development but the mechanisms underlying progression of gliomas to malignancy are by no means established.[1] It has become increasingly apparent that cooperation between signaling pathways induced by growth factors and cytokines and those induced by cell-cell and cell-extracellular matrix interactions are central to regulation of cell behavior. Recent work has highlighted the importance of altered cell-matrix interactions in the acquisition of malignant characteristics.[2–4] Hyaluronan is a very large, negatively charged polysaccharide composed of repeating units of glucuronic acid and *N*-acetylglucosamine. It is ubiquitously associated with extracellular and pericellular matrices but is especially enriched around proliferating and migrating cells.[5] Interactions of hyaluronan with a variety of cell-surface receptors activate intracellular signaling pathways that regulate various aspects of cell behavior[6] and, in tumor cells, promote malignant characteristics.[7–9]

Several studies have provided evidence that hyaluronan-cell interactions may play a role in glioma invasiveness. For example, addition of exogenous hyaluronan enhances glioma cell migration and invasion *in vitro*, and this effect is blocked by antibodies or anti-sense oligonucleotides to CD44, a cell-surface hyaluronan receptor.[10–13] Also, hyaluronan stimulates extracellular-regulated protein kinase phosphorylation and up-regulates components of the plasminogen activation cascade in glioma cells as a result of interactions between CD44 and the epidermal growth factor receptor.[14] However, most of these studies address effects of exogenously added hyaluronan rather than endogenous hyaluronan-cell interactions. This is an important distinction because hyaluronan interacts with cells in a variety of ways, some of which are not necessarily duplicated by addition of exogenous hyaluronan.[5,8] For example, nascent hyaluronan is often retained at the cell surface by sustained transmembrane

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interaction with hyaluronan synthases on the inner side of the plasma membrane,[15–17] an arrangement that is not mimicked by exogenous hyaluronan. Also, hyaluronan mediates assembly of complex pericellular matrices containing several components.[5,18,19] Although the latter can be reconstituted in part by the addition of exogenous components,[19,20] it is unlikely that the complete native configuration of these matrices can be duplicated in this way. In the present study we examine the effects of perturbing endogenous hyaluronan-cell interactions on glioma cell growth and invasion, using two different approaches. The first is addition of hyaluronan oligomers that competitively displace endogenous polymer from its receptors. This leads to low-affinity, monovalent binding rather than high-affinity, polyvalent binding[21,22] and thus to attenuated signaling.[23] The second approach is overexpression of soluble hyaluronan-binding proteins (HABPs) that compete with endogenous receptors by binding endogenous hyaluronan.[24–29] We find that both manipulations cause inhibition of anchorage-independent growth and invasion of glioma cells, indicating that endogenous hyaluronan interactions are required for these two hallmark characteristics of malignant glioma cells.

Materials and Methods

Cells and Reagents

C6 rat glioma cells, A172 human glioma cells, and U87 human glioma cells were obtained from the American Type Culture Collection (Rockville, MD). C6 and A172 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Grand Island, NY) and U87 cells in minimal essential medium (Life Technologies, Inc.). Media contained 10% fetal bovine serum (Hyclone, Logan, UT) and penicillin/streptomycin (Life Technologies, Inc.) unless otherwise stated.

Highly purified hyaluronan oligomers were fractionated from testicular hyaluronidase digests of hyaluronan polymer by tangential flow filtration as described previously[30] and were donated by Anika Therapeutics Inc. (Woburn, MA). These oligomers were a mixture of average molecular weight $\sim 2.5 \times 10^3$ ($\sim$3 to 10 disaccharide units). They were analyzed by high performance liquid chromatography and capillary electrophoresis but no contaminants were detected. Specific analyses for other glycosaminoglycans, protein, nucleic acids, and endotoxins were negative.

Chitin oligomers were obtained from Seikagaku America (Falmouth, MA). *N*-acetylglucosamine and glucuronic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies against phosphorylated Akt and hemoagglutinin tag were from Pharmingen (La Jolla, CA) and Boehringer-Mannheim (Indianapolis, IN), respectively. Antibodies against CD44 were from Pharmingen or Zymed (South San Francisco, CA). Mouse IgG, rat IgG, and rabbit IgG were from Calbiochem (La Jolla, CA), BD Biosciences (San Diego, CA) and Oncogene Research Products (San Diego, CA), respectively.

Recombinant HABP Adenoviruses

The cDNA for soluble CD44 contained the signal peptide domain and single hyaluronan-binding, link module domain that are common to all forms of CD44, plus variant exon products v6, v7, v8, v9, and truncated v10, as described previously.[31] This construct lacks the C-terminal portion of the extracellular domain, the transmembrane domain and the cytoplasmic domain, thus giving rise to a soluble, secreted product.[31] The cDNA for brevican link module consists of the signal peptide, Ig fold, and the two link modules that comprise the hyaluronan-binding domain of rat brevican (amino acids 1 to 363, with a hemagglutinin tag added at amino acid position 364).[32,33] The two cDNAs were cloned into the pACCM-V.pLpA shuttle vector. Each construct was then co-transfected into 293 cells with the pJM17 adenovirus using a calcium phosphate co-immunoprecipitation protocol as described previously.[34,35] Plaques resulting from successful homologous recombination were then chosen, amplified, and purified using cesium chloride gradient centrifugation.[34] The titer was typically 10^{12} pfu/ml. A β-galactosidase recombinant adenovirus was used as a negative control. This virus and the vectors for preparation of the HABP adenoviruses were kindly donated by Dr. Kenneth Walsh, Boston University, Boston, MA.

For infection, C6 cells were plated in 100-mm dishes at 80 to 90% confluency, then washed with serum-free DMEM media. Five ml of serum-free DMEM medium was then added to the cells followed by $\sim$200 to 300 viral particles/cell. After 30 minutes with occasional rocking, the medium was removed and replaced with 5 ml of fresh serum-free DMEM media. After 24 hours, the cells were harvested for analysis of protein expression or for use in the soft agar and invasion assays.

For analysis of protein expression, the cells were washed twice with ice-cold phosphate-buffered saline (PBS). Extraction buffer (50 mmol/L Tris-HCL, pH 7.4, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 2 mmol/L phenylmethyl sulfonyl fluoride, 1 μg/ml leupeptin, and 1.0 U/ml aprotinin) was then added to the cells. The cells were harvested by scraping, added to a 1.5-ml microcentrifuge tube, placed on ice for 20 minutes, centrifuged at $14{,}000 \times g$ for 30 minutes at 4°C, and the cell extract was then removed for analysis. The media and cell extract were run on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred to nitrocellulose, and probed with an anti-CD44 (Pharmingen) or anti-hemoagglutinin antibody.

Soft Agar Assay

The bottom of each well of a six-well plate (CoStar, Cambridge, MA) was layered with 1.2 ml of 0.6% agarose (Life Technologies, Inc.) in DMEM, obtained by mixing equal volumes of 1.2% agarose with 2× DMEM at 37°C then incubating at 4°C. Once solidified, the 0.6% agarose was covered with 1.2 ml of 0.1% agarose in DMEM solution containing 2×10^3 C6 rat glioma cells per well, with or

without addition of hyaluronan oligomers or other additives. Each condition was assayed in triplicate. The plates were placed at 4°C, for 15 minutes to allow the top layer of agarose to completely solidify, then allowed to equilibrate to room temperature followed by incubation at 37°C, 5.0% CO_2, for 1 week. The number of colonies obtained under each condition was counted using an ocular scale (10x/18l; Olympus, Tokyo, Japan). Colonies larger than 0.25 mm in diameter were counted. The total number of colonies formed in each case is compared using the Student's *t*-test.

Invasion Chamber Assay

The glioma cells (in serum-free media) were placed on inserts, containing 8-micron pores and coated with reconstituted basement membrane matrix (Matrigel; Becton-Dickinson, Mountain View, CA). The coated inserts were placed between the two chambers of wells within an invasion apparatus (Becton-Dickinson). A chemoattractant consisting of 10% fetal bovine serum (Hyclone) and 10 μg/ml fibronectin (Collaborative-Biomedical Products, Bedford, MA) was added to the lower chamber. Triplicate wells containing 1 to 2 × 10^4 of C6, A172, or U87 cells per well, in the presence or absence of hyaluronan oligomers or other reagents as described in the text, were used in each experiment. Unless otherwise stated, the cells were incubated in the invasion chambers for 24 hours. The inserts were then removed and inverted. The cells that had invaded through the inserts were fixed for 1 minute with glutaraldehyde, stained for 5 minutes with a 0.1% cresyl violet solution, and counted using a dissecting microscope. The total number of cells invaded in each case is compared using the Student's *t*-test.

Western Blot Analysis of Phosphorylated Akt

C6 glioma cells were grown in suspension in six-well Ultra-Low Cluster (ULC) plates (CoStar). Approximately 10^5 C6 cells were plated in DMEM (Life Technologies, Inc.) in each well. After 48 hours, hyaluronan oligomers, chitin oligomers, or hyaluronan polymer (~80 kd; Genzyme, Cambridge, MA) in PBS, or PBS alone, were added to the cells. After another 24 hours, the cells were collected, lysed, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, using antibody against phospho-Akt and Akt (BD Pharmingen).

Results

Hyaluronan Oligomers Inhibit Glioma Cell Invasion

Oligomers of hyaluronan compete for binding of polymeric hyaluronan to cell-surface receptors, resulting in low-affinity binding[21,22] and attenuation of hyaluronan-induced signaling.[23] Thus we tested whether hyaluronan oligomers inhibit glioma cell invasion of reconstituted basement membrane gels. We found that, at a concentration of 100 μg/ml, hyaluronan oligomers inhibit invasion of these gels by C6 rat glioma cells to the extent of 70 to 90% throughout the course of at least 72 hours (Figure 1A). The inhibitory effect of the oligomers occurs at concentrations as low as 1 μg/ml (Figure 1B); some inhibition may have occurred below this concentration but it was not statistically significant (not shown). We compared the inhibitory effect of 100 μg/ml of the oligomers on three different glioma cell lines, ie, C6 rat glioma cells, A172 human glioma cells, and U87 human glioma cells, throughout a 24-hour period and obtained ~80%, 70%, and 90% inhibition, respectively (Figure 1B). This experiment was repeated three times with each cell type and similar results were obtained in each experiment. As a control we also tested the effect of chito-oligosaccharides of approximately the same size as the hyaluronan oligomers. We used chitin oligomers because they are composed of repeating units of *N*-acetylglucosamine, a component of hyaluronan oligomers; they

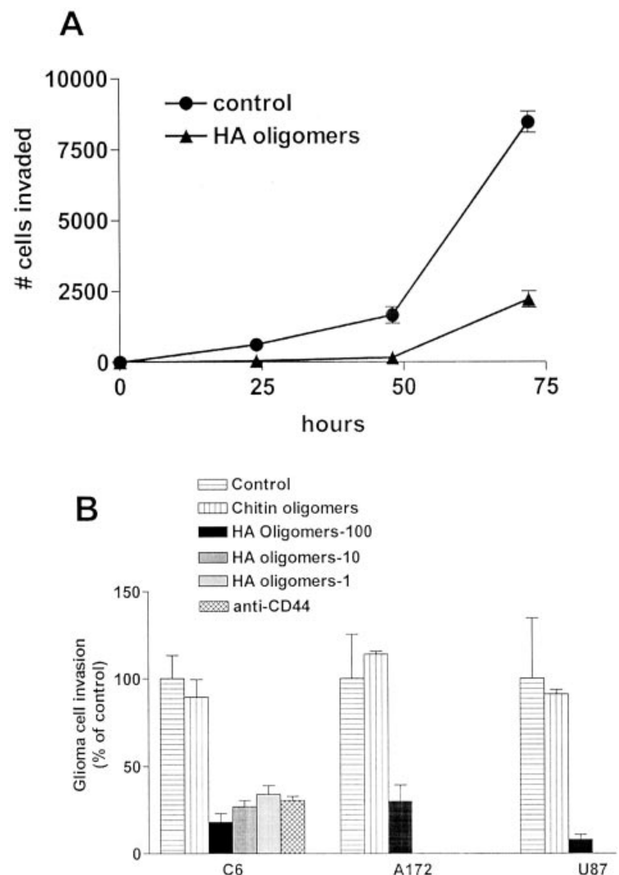

Figure 1. Inhibition of glioma cell invasion by hyaluronan oligomers. **A:** C6 glioma cells (2 × 10^4 per well) were incubated in the presence or absence of 100 μg/ml of hyaluronan (HA) oligomers for 72 hours and the number of cells that invaded the reconstituted basement membrane matrix was measured at each 24-hour interval, as described in Materials and Methods. **B:** C6 rat glioma cells (2 × 10^4 per well), A172 human glioma cells (1 × 10^4 per well), and U87 human glioma cells (1 × 10^4 per well) were incubated in the presence and absence of 1 to 100 μg/ml of hyaluronan oligomers, 100 μg/ml of chitin oligomers, or 4 μg/ml of antibody against CD44 (Zymed), and the number of cells that invaded was measured after 24 hours. The results in **B** are presented as means of three experiments ± SD (control *versus* hyaluronan oligomers; $P < 0.05$ for each cell type).

are sufficiently similar to hyaluronan that hyaluronan synthases are capable of producing chitin oligosaccharides as well as hyaluronan.[36] Despite this similarity, the chitin oligomers have no significant effect on invasion of reconstituted basement membrane matrix by C6, A172, or U87 cells (Figure 1B). Thus hyaluronan oligomers are potent and specific inhibitors of glioma cell invasiveness.

As discussed above, hyaluronan oligomers most likely act by displacing endogenous hyaluronan polymer from its cell-surface receptor(s). Since past studies suggest that the hyaluronan receptor, CD44, is involved in glioma invasion, we tested the effect of a function-blocking antibody against CD44 and found that it inhibited glioma cell invasion by 60 to 70% (Figure 1B) in agreement with the results of other groups.[10–13]

To ensure that the hyaluronan oligomers are not toxic to glioma cells, we examined their effect on growth in routine monolayer culture. Treatment of C6, A172, or U87 cells throughout a 72-hour time period with 100 μg/ml of oligomers had no significant effect on proliferation. We also found that the glioma cells continued to proliferate at a high rate in the invasion chambers, such that after 4 to 5 days of incubation more cells penetrated the reconstituted matrix, in the presence or absence of hyaluronan oligomers, than were placed in the chambers at the beginning of the experiment (data not shown). We also investigated whether hyaluronan oligomers inhibit cell migration, as distinct from cell invasion through extracellular matrix. For this, we used 24-well, 8.0-micron pore inserts, similar to those used in the invasion assays, but the wells were not coated with reconstituted basement membrane matrix. We observed that the hyaluronan oligomers did not inhibit C6 or A172 cell migration throughout a 72-hour period whereas they had a moderate but significant inhibitory effect on U87 cell migration (not shown).

Overexpression of Soluble HABPs Inhibits Glioma Cell Invasion

To confirm that perturbation of hyaluronan interactions inhibits glioma cell invasion, we examined the effect of overexpression of two soluble HABPs, ie, soluble CD44 and brevican link module. These soluble HABPs act as decoys by binding endogenous hyaluronan,[24–29] thus inhibiting its downstream effects. For overexpression, we produced recombinant HABP adenoviruses and verified that these constructs successfully drive production of the HABPs after infection of C6 cells with the adenoviruses. Cell extracts and media were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, using appropriate antibodies. We observed that infection with the soluble CD44 or brevican link module adenoviral construct induced their production and that in each case a large proportion of the HABP was secreted (Figure 2, insets). Then, to determine whether expression of the soluble HABPs affects glioma cell invasion, we infected C6 cells with either the soluble CD44 or the brevican link module adenoviral construct and tested their ability to invade reconstituted basement

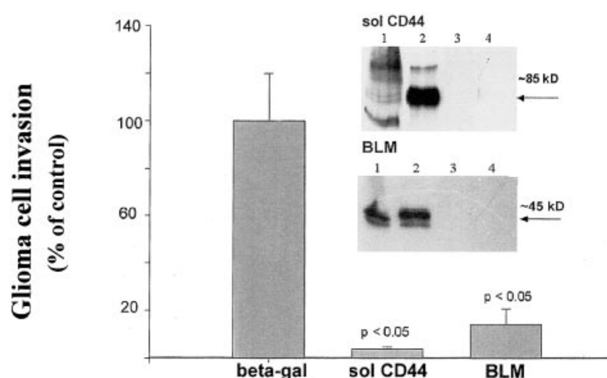

Figure 2. Inhibition of C6 glioma cell invasion by soluble HABPs. C6 cells were infected with recombinant adenoviruses driving expression of β-galactosidase (β-gal), soluble CD44 (solCD44), or brevican link module (BLM), then assayed for invasion as in Figure 1B. The **insets** show expression of the HABPs, as measured by Western blotting. **Top inset**: **lanes 1** and **2,** soluble CD44 adenovirus; **lanes 3** and **4**, β-galactosidase adenovirus; **lanes 1** and **3**, cell extract; **lanes 2** and **4**, medium. Western blotting was performed with antibody to CD44. **Bottom inset**: **lanes 1** and **2**, brevican link module adenovirus; **lanes 3** and **4**, β-galactosidase adenovirus; **lanes 1** and **3**, cell extract; **lanes 2** and **4**, medium. Western blotting was performed with antibody to the hemoagglutinin tag attached to BLM.

membrane gels, using a β-galactosidase construct as a negative control. As seen in Figure 2, both of the soluble HABPs caused dramatic inhibition of invasion by the C6 cells.

Perturbation of Hyaluronan Interactions Inhibits Anchorage-Independent Growth of Glioma Cells

As described above, we found that hyaluronan oligomers, at the concentrations used in this study, do not have a significant effect on growth in regular monolayer culture. We then determined the effect of hyaluronan oligomers on the ability of C6 glioma cells to form colonies in soft agar, which is a common method of assessing anchorage-independent growth, a characteristic that distinguishes most transformed and tumor cells from normal cells.[37] In several independent experiments, addition of hyaluronan oligomers was found to inhibit formation of colonies by ~40 to 60% at concentrations between 10 and 100 μg/ml (Figure 3A). Addition of equivalent amounts of the two monosaccharide subunits of hyaluronan, ie, *N*-acetylglucosamine and glucuronic acid, did not inhibit colony formation.

Previous results obtained in our laboratory indicate that hyaluronan oligomers inhibit anchorage-independent growth of mammary and colon carcinoma cells by suppressing the phosphoinositide-3-kinase (PI3K)/Akt cell survival pathway.[23] In three separate experiments, we found that addition of 100 μg/ml of oligomers caused >95% inhibition of phosphorylation of Akt in C6 glioma cells (Figure 3B), in similar manner to that found with other tumor cell types.[23] Similar treatment had an insignificant to modest effect (0 to 35% inhibition throughout three experiments) on total Akt levels. Chitin oligomers and hyaluronan polymer (~80 kD) did not have significant effects on total or phosphorylated Akt levels in any of the three experiments (Figure 3B).

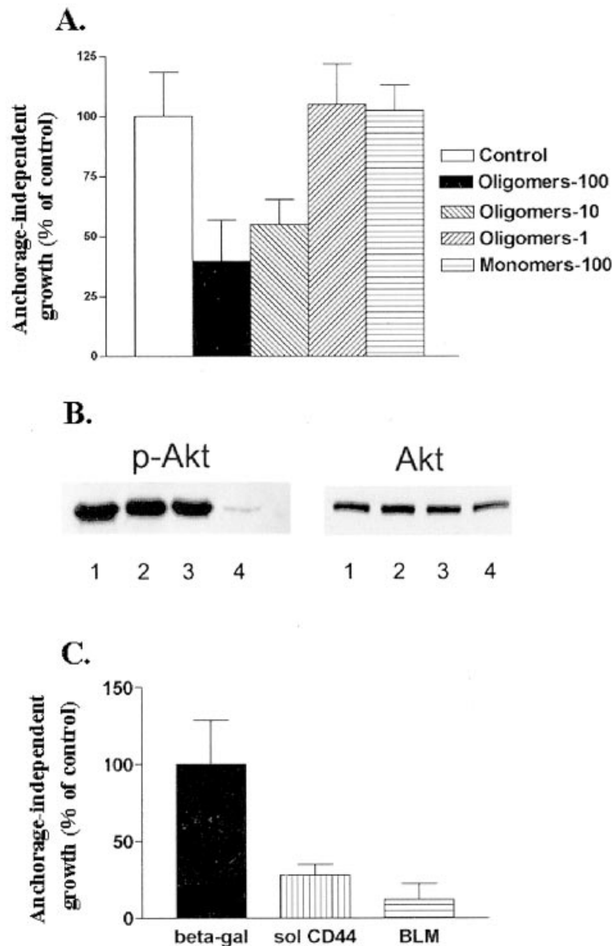

Figure 3. Inhibition of anchorage-independent growth of C6 glioma cells by hyaluronan oligomers or by overexpression of soluble HABPs. **A:** C6 cells (2×10^3 per well) were incubated for 1 week in soft agar in the presence or absence of 1, 10, or 100 μg/ml of hyaluronan oligomers, or 50 μg/ml of *N*-acetylglucosamine plus 50 μg/ml of glucuronic acid monomers, as described in Materials and Methods. The numbers of colonies >0.25 mm in size were counted. The results are presented as means of triplicates ± SD (control *versus* 10 or 100 μg/ml hyaluronan oligomers; $P < 0.05$). **B:** Western blot with antibody against phosphorylated Akt (p-Akt) or Akt for extracts of C6 cells grown in suspension. **Lane 1**, C6 cells alone; **lane 2**, C6 cells incubated with 100 μg/ml of chitin oligomers; **lane 3**, with 100 μg/ml of hyaluronan polymer (~80 kD); **lane 4**, with 100 μg/ml of hyaluronan oligomers. **C:** C6 cells (2×10^3 per well) were infected with recombinant adenoviruses driving expression of β-galactosidase (β-gal), soluble CD44 (solCD44), or brevican link module (BLM), then assayed for growth of colonies in soft agar. The results are presented as means of three separate experiments, each performed in triplicate, ± SD (control *versus* soluble CD44 or brevican link module; $P < 0.05$).

To further test the effect of perturbing hyaluronan-tumor cell interactions on anchorage-independent growth, we used recombinant adenoviral constructs driving expression of soluble CD44 or brevican link module in C6 cells. We found that overexpression of either of these HABPs reduces the number of colonies formed in soft agar (Figure 3C). Three separate experiments were performed and inhibition varied from ~40 to 90%.

Discussion

In this study, two types of manipulation were used to perturb hyaluronan-glioma cell interactions: treatment with hyaluronan oligomers and overexpression of soluble HABPs. The results obtained indicate that perturbation of these interactions inhibits two distinguishing characteristics of malignant tumor cells, ie, invasiveness and anchorage-independent growth. We conclude from these findings that endogenous hyaluronan interactions are necessary for these properties of malignant glioma cells.

Hyaluronan oligomers compete for endogenous hyaluronan polymer-cell interactions, resulting in low-valency, low-affinity binding. Previous work from this laboratory has demonstrated that hyaluronan oligomers inhibit tumor growth *in vivo*[23,30] and suppress the PI3K/Akt cell survival pathway, thus inducing apoptosis in carcinoma cells under anchorage-independent growth conditions.[23] In agreement with these results, we find here that treatment with hyaluronan oligomers inhibits colony formation in soft agar and decreases the level of phosphorylated Akt in glioma cells. Thus it seems that hyaluronan is necessary for this malignant growth characteristic of glioma cells.

Treatment with hyaluronan oligomers also inhibits glioma cell invasion. Studies from other laboratories have shown that the PI3K pathway is required for tumor cell invasion,[38–40] and thus suppression of the PI3K pathway may underlie the effect of hyaluronan oligomers on glioma invasion as well as on anchorage-independent growth. Inhibition of invasion was not because of induction of apoptosis because, under the anchorage-dependent conditions of the invasion assay, cell proliferation continues at a high rate in the presence or absence of the oligomers. Also, cell movement, which is necessary for invasion, was not affected significantly by the hyaluronan oligomers in two of the three invasive glioma cells tested. Another parameter that is important for invasion is production of matrix metalloproteinases,[41–43] whose production is regulated by the PI3K pathway[38–40] and is stimulated by treatment with polymeric hyaluronan.[44,45] In glioma cells, hyaluronan stimulation of gelatinase B is inhibited by the tumor suppressor, PTEN, at least in part because of inactivation of focal adhesion kinase rather than the PI3K pathway.[45] We have not observed a significant effect of the oligomers on endogenous matrix metalloproteinase levels in our studies. However, we have found that treatment with hyaluronan oligomers reverses the induction of gelatinase A production caused by addition of exogenous polymeric hyaluronan. Possibly, the oligomers cause a more subtle effect on distribution or activation of endogenous MMPs, eg, interference with docking of gelatinase B or matrilysin to CD44.[38,46,47]

Several studies, including ours, have implicated the hyaluronan receptor, CD44, in glioma invasion. However we, and others,[10,11,13] do not obtain complete inhibition of invasion by suppressing CD44 binding, suggesting either that other hyaluronan receptors are involved or that hyaluronan has effects that are independent of cell-surface receptors. In agreement with the former possibility, a recent study demonstrated that interaction of hyaluronan with another receptor, RHAMM, is involved in the effects of hyaluronan on glioma cell proliferation and migration.[48] However, hyaluronan may also promote cell invasion of

extracellular matrices in a receptor-independent manner by creating a malleable matrix with hydrated spaces between structural barriers to invasion.[5,49] Thus it is conceivable that hyaluronan oligomers inhibit invasion because of interference with interactions between endogenous hyaluronan and other matrix components that are necessary for the integrity of such a hydrated matrix. The variability of results obtained in different laboratories with different glioma cell types suggests that the relative contribution of receptor-mediated and receptor-independent effects of hyaluronan on cell invasion may depend on the status and source of the glioma cells.

The second manipulation found to inhibit glioma cell invasion and anchorage-independent growth in this study was overexpression of soluble HABPs. The two soluble HABPs used here were the ectodomain of a CD44 variant and the hyaluronan-binding link module domains of the proteoglycan, brevican. Overexpression was achieved by infection of C6 cells with recombinant adenoviruses driving expression of these soluble HABPs. Soluble HABPs compete for binding of endogenous hyaluronan to cell-surface receptors and consequently would prevent downstream signaling. Previous studies have demonstrated that overexpression of soluble CD44 via stable transfection inhibits tumor growth, invasion, and metastasis *in vivo*.[24–26,28] Similar results have been obtained with other soluble HABPs, eg, soluble RHAMM,[27] and HABPs extracted from cartilage.[29] As is the case for the hyaluronan oligomers, the inhibitory effects of soluble HABPs may derive from more than one mechanism. For example, soluble CD44 induces apoptosis *in vivo*,[25] presumably via suppression of the PI3K pathway in similar manner to the effect of hyaluronan oligomers.[23] However, the effect of reduced PI3K on apoptosis would only be seen *in vitro* under anchorage-independent conditions, eg, in the soft agar colony assay.[23] The second possible mode of action of soluble HABPs is interference with docking of gelatinase B to endogenous cell-surface CD44, thus blocking invasion and processing of transforming growth factor-β.[46,50] This latter mechanism may also be secondary to suppression of the PI3K pathway.[38] In addition, however, soluble HABPs may affect receptor-independent influences of hyaluronan on cell invasion in a similar manner to that discussed above in relation to the hyaluronan oligomers.[5,49]

It will be of considerable interest to determine whether perturbation of hyaluronan-glioma cell interactions by the methods used herein inhibit glioma progression *in vivo*, and whether interventions based on these approaches might provide useful therapies in glioma patients.

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References

1. Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK, DePinho RA: Malignant glioma: genetics and biology of a grave matter. Genes Dev 2001, 15:1311–1333
2. Bissell MJ, Weaver VM, Lelievre SA, Wang F, Petersen OW, Schmeichel KL: Tissue structure, nuclear organization, and gene expression in normal and malignant breast. Cancer Res 1999, 59: 1757s–1764s
3. Sternlicht MD, Werb Z: How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001, 17:463–516
4. Yamada KM, Araki M: Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. J Cell Sci 2001, 114: 2375–2382
5. Toole BP: Hyaluronan in morphogenesis. Semin Cell Dev Biol 2001, 12:79–87
6. Turley EA, Noble PW, Bourguignon LY: Signaling properties of hyaluronan receptors. J Biol Chem 2002, 277:4589–4592
7. Toole BP, Wight TN, Tammi M: Hyaluronan-cell interactions in cancer and vascular disease. J Biol Chem 2002, 277:4593–4596
8. Toole BP: Hyaluronan promotes the malignant phenotype. Glycobiology 2002, 12:37R–42R
9. Toole BP, Hascall VC: Hyaluronan and tumor growth. Am J Pathol 2002, 161:745–747
10. Merzak A, Koocheckpour S, Pilkington GJ: CD44 mediates human glioma cell adhesion and invasion in vitro. Cancer Res 1994, 54: 3988–3992
11. Koochekpour S, Pilkington GJ, Merzak A: Hyaluronic acid/CD44H interaction induces cell detachment and stimulates migration and invasion of human glioma cells in vitro. Int J Cancer 1995, 63:450–454
12. Okada H, Yoshida J, Sokabe M, Wakabayashi T, Hagiwara M: Suppression of CD44 expression decreases migration and invasion of human glioma cells. Int J Cancer 1996, 66:255–260
13. Radotra B, McCormick D: CD44 is involved in migration but not spreading of astrocytoma cells in vitro. Anticancer Res 1997, 17:945–949
14. Tsatas D, Kanagasundaram V, Kaye A, Novak U: EGF receptor modifies cellular responses to hyaluronan in glioblastoma cell lines. J Clin Neurosci 2002, 9:282–288
15. Heldin P, Pertoft H: Synthesis and assembly of the hyaluronan-containing coats around normal human mesothelial cells. Exp Cell Res 1993, 208:422–429
16. Nishida Y, Knudson CB, Nietfeld JJ, Margulis A, Knudson W: Antisense inhibition of hyaluronan synthase-2 in human articular chondrocytes inhibits proteoglycan retention and matrix assembly. J Biol Chem 1999, 274:21893–21899
17. Spicer AP, McDonald JA: Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. J Biol Chem 1998, 273:1923–1932
18. Tammi MI, Day AJ, Turley EA: Hyaluronan and homeostasis: a balancing act. J Biol Chem 2002, 277:4581–4584
19. Knudson CB, Nofal GA, Pamintuan L, Aguiar DJ: The chondrocyte pericellular matrix: a model for hyaluronan-mediated cell-matrix interactions. Biochem Soc Trans 1999, 27:142–147
20. Knudson W, Bartnik E, Knudson CB: Assembly of pericellular matrices by COS-7 cells transfected with CD44 lymphocyte-homing receptor genes. Proc Natl Acad Sci USA 1993, 90:4003–4007
21. Underhill CB, Chi-Rosso G, Toole BP: Effects of detergent solubilization on the hyaluronate-binding protein from membranes of simian virus 40-transformed 3T3 cells. J Biol Chem 1983, 258:8086–8091
22. Lesley J, Hascall VC, Tammi M, Hyman R: Hyaluronan binding by cell surface CD44. J Biol Chem 2000, 275:26967–26975
23. Ghatak S, Misra S, Toole BP: Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/Akt cell survival pathway. J Biol Chem 2002, 277:38013–38020
24. Bartolazzi A, Peach R, Aruffo A, Stamenkovic I: Interaction between CD44 and hyaluronate is directly implicated in the regulation of tumor development. J Exp Med 1994, 180:53–66
25. Yu Q, Toole BP, Stamenkovic I: Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. J Exp Med 1997, 186:1985–1996

26. Peterson RM, Yu Q, Stamenkovic I, Toole BP: Perturbation of hyaluronan interactions by soluble CD44 inhibits growth of murine mammary carcinoma cells in ascites. Am J Pathol 2000, 156:2159–2167
27. Mohapatra S, Yang X, Wright JA, Turley EA, Greenberg AH: Soluble hyaluronan receptor RHAMM induces mitotic arrest by suppressing Cdc2 and cyclin B1 expression. J Exp Med 1996, 183:1663–1668
28. Ahrens T, Sleeman JP, Schempp CM, Howells N, Hofmann M, Ponta H, Herrlich P, Simon JC: Soluble CD44 inhibits melanoma tumor growth by blocking cell surface CD44 binding to hyaluronic acid. Oncogene 2001, 20:3399–3408
29. Liu N, Lapcevich RK, Underhill CB, Han Z, Gao F, Swartz G, Plum SM, Zhang L, Gree SJ: Metastatin: a hyaluronan-binding complex from cartilage that inhibits tumor growth. Cancer Res 2001, 61:1022–1028
30. Zeng C, Toole BP, Kinney SD, Kuo JW, Stamenkovic I: Inhibition of tumor growth in vivo by hyaluronan oligomers. Int J Cancer 1998, 77:396–401
31. Yu Q, Toole BP: A new alternatively spliced exon between v9 and v10 provides a molecular basis for synthesis of soluble CD44. J Biol Chem 1996, 271:20603–20607
32. Jaworski DM, Kelly GM, Hockfield S: BEHAB, a new member of the proteoglycan tandem repeat family of hyaluronan-binding proteins that is restricted to the brain. J Cell Biol 1994, 125:495–509
33. Yamada H, Watanabe K, Shimonaka M, Yamaguchi Y: Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family. J Biol Chem 1994, 269:10119–10126
34. Becker TC, Noel RJ, Coats WS, Gomez-Foix AM, Alam T, Gerard RD, Newgard CB: Use of recombinant adenovirus for metabolic engineering of mammalian cells. Methods Cell Biol 1994, 43:161–189
35. Li R, Huang L, Guo H, Toole BP: Basigin (murine EMMPRIN) stimulates matrix metalloproteinase production by fibroblasts. J Cell Physiol 2001, 186:371–379
36. Yoshida M, Itano N, Yamada Y, Kimata K: In vitro synthesis of hyaluronan by a single protein derived from mouse HAS1 gene and characterization of amino acid residues essential for the activity. J Biol Chem 2000, 275:497–506
37. Freedman VH, Shin SI: Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell 1974, 3:355–359
38. Ellerbroek SM, Halbleib JM, Benavidez M, Warmka JK, Wattenberg EV, Stack MS, Hudson LG: Phosphatidylinositol 3-kinase activity in epidermal growth factor-stimulated matrix metalloproteinase-9 production and cell surface association. Cancer Res 2001, 61:1855–1861
39. Kim D, Kim S, Koh H, Yoon SO, Chung AS, Cho KS, Chung J: Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. EMBO J 2001, 15:1953–1962
40. Kubiatowski T, Jang T, Lachyankar MB, Salmonsen R, Nabi RR, Quesenberry PJ, Litofsky NS, Ross AH, Recht LD: Association of increased phosphatidylinositol 3-kinase signaling with increased invasiveness and gelatinase activity in malignant gliomas. J Neurosurg 2001, 95:480–488
41. Koul D, Parthasarathy R, Shen R, Davies MA, Jasser SA, Chintala SK, Rao JS, Sun Y, Benvenisite EN, Liu TJ, Yung WK: Suppression of matrix metalloproteinase-2 gene expression and invasion in human glioma cells by MMAC/PTEN. Oncogene 2001, 20:6669–6678
42. Lakka SS, Jasti SL, Kyritsis AP, Yung WK, Ali-Osman F, Nicolson GL, Rao JS: Regulation of MMP-9 (type IV collagenase) production and invasiveness in gliomas by the extracellular signal-regulated kinase and jun amino-terminal kinase signaling cascades. Clin Exp Metastasis 2000, 18:245–252
43. Watanabe K, Yoshida D, Noha M, Teramoto A: Suppression of matrix metalloproteinase-2 and -9 mediated invasiveness by a novel matrix metalloproteinase inhibitor, BE16627B. J Neurooncol 2001, 52:1–9
44. Zhang Y, Thant AA, Machida K, Ichigotani Y, Naito Y, Hiraiwa Y, Senga T, Sohara Y, Matsuda S, Hamaguchi M: Hyaluronan-CD44s signaling regulates matrix metalloproteinase-2 secretion in a human lung carcinoma cell line QG90. Cancer Res 2002, 62:3962–3965
45. Park MJ, Kim MS, Park IC, Kang HS, Yoo H, Park SH, Rhee CH, Hong SI, Lee SH: PTEN suppresses hyaluronic acid-induced matrix metalloproteinase-9 expression in U87MG glioblastoma cells through focal adhesion kinase dephosphorylation. Cancer Res 2002, 62:6318–6322
46. Yu Q, Stamenkovic I: Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes Dev 1999, 13:35–48
47. Yu WH, Woessner Jr JF, McNeish JD, Stamenkovic I: CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. Genes Dev 2002, 16:307–323
48. Akiyama Y, Jung S, Salhia B, Lee S, Hubbard S, Taylor M, Mainprize T, Akaishi K, van Furth W, Rutka JT: Hyaluronate receptors mediating glioma cell migration and proliferation. J Neurooncol 2001, 53:115–127
49. Hayen W, Goebeler M, Kumar S, Riessen R, Nehls V: Hyaluronan stimulates tumor cell migration by modulating the fibrin fiber architecture. J Cell Sci 1999, 112:2241–2251
50. Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000, 14:163–176