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.5–4 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 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, an arrangement that is not mimicked by exogenous hyaluronan. Also, hyaluronan mediates assembly of complex pericellular matrices containing several components. Although the latter can be reconstituted in part by the addition of exogenous components, 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 and thus to attenuated signaling. The second approach is overexpression of soluble hyaluronan-binding proteins (HABPs) that compete with endogenous receptors by binding endogenous hyaluronan. 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.). 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. The second approach is overexpression of soluble hyaluronan-binding proteins (HABPs) that compete with endogenous receptors by binding endogenous hyaluronan. 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.

**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. 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 (Phar mingon) or anti-hemoagglutinin antibody.

**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. 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. 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). The two cDNAs were cloned into the pACCMVpLPAd shuttle vector. Each construct was then co-transformed into 293 cells with the pJM17 adenovirus using a calcium phosphate co-immunoprecipitation protocol as described previously. Plaques resulting from successful homologous recombination were then chosen, amplified, and purified using cesium chloride gradient centrifugation. 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 ~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 × 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 (Phar mingon) or anti-hemoagglutinin antibody.

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Highly purified hyaluronan oligomers were fractionated from testicular hyaluronidase digests of hyaluronan polymer by tangential flow filtration as described previously and were donated by Anika Therapeutics Inc. (Woburn, MA). These oligomers were a mixture of average molecular weight ~2.5 × 10^6 (~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 Phar mingon 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.
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% CO₂, for 1 week. The number of colonies obtained under each condition was counted using an ocular scale (10x/18; 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 x 10⁴ 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 10⁵ 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 and attenuation of hyaluronan-induced signaling. 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 chitooligosaccharides 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
are sufficiently similar to hyaluronan that hyaluronan synthases are capable of producing chitin oligosaccharides as well as hyaluronan. 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. To confirm that perturbation of hyaluronan interactions inhibits glioma cell invasion, 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, 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 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. 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. 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. 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).
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-independence 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 vivo23,30 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 pathway38–40 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.46 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 matrixins 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 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.\textsuperscript{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\textit{ in vivo}.\textsuperscript{24–26,28} Similar results have been obtained with other soluble HABPs, eg, soluble RHAMM\textsuperscript{27} and HABPs extracted from cartilage.\textsuperscript{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 \textit{in vivo},\textsuperscript{25} presumably via suppression of the PI3K pathway in similar manner to the effect of hyaluronan oligomers.\textsuperscript{23} However, the effect of reduced PI3K on apoptosis would only be seen \textit{in vitro} under anchorage-independent conditions, eg, in the soft agar colony assay.\textsuperscript{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-$\beta$.\textsuperscript{46,50} This latter mechanism may also be secondary to suppression of the PI3K pathway.\textsuperscript{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.\textsuperscript{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 \textit{in vivo}, and whether interventions based on these approaches might provide useful therapies in glioma patients.

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\section*{References}