Targeting Hyaluronan Interactions in Malignant Gliomas and Their Drug-Resistant Multipotent Progenitors

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Abstract

**Purpose:** To determine if hyaluronan oligomers (o-HA) antagonize the malignant properties of glioma cells and treatment-resistant glioma side population (SP) cells in vitro and in vivo.

**Experimental Design:** A single intratumoral injection of o-HA was given to rats bearing spinal cord gliomas 7 days after engraftment of C6 glioma cells. At 14 days, spinal cords were evaluated for tumor size, invasive patterns, proliferation, apoptosis, activation of Akt, and BCRP expression. C6SP were isolated by fluorescence-activated cell sorting and tested for the effects of o-HA on BCRP expression, activation of Akt and epidermal growth factor receptor, drug resistance, and glioma growth in vivo.

**Results:** o-HA treatment decreased tumor cell proliferation, increased apoptosis, and down-regulated activation of Akt and the expression of BCRP. o-HA treatment of C6SP inhibited activation of epidermal growth factor receptor and Akt, decreased BCRP expression, and increased methotrexate cytotoxicity. In vivo, o-HA also suppressed the growth of gliomas that formed after engraftment of C6 or BCRP+ C6SP cells, although most C6SP cells lost their expression of BCRP when grown in vivo. Interestingly, the spinal cord gliomas contained many BCRP+ cells that were not C6 or C6SP cells but that expressed nestin and/or CD45; o-HA treatment significantly decreased the recruitment of these BCRP+ progenitor cells into the engrafted gliomas.

**Conclusions:** o-HA suppress glioma growth in vivo by enhancing apoptosis, down-regulating key cell survival mechanisms, and possibly by decreasing recruitment of host-derived BCRP+ progenitor cells. Thus, o-HA hold promise as a new biological therapy to inhibit HA-mediated malignant mechanisms in gliomas and treatment-resistant glioma stem cells.

Malignant gliomas are common central nervous system (CNS) tumors, and regrettably, the most aggressive subtype glioblastoma multiforme makes up >50% of newly diagnosed cases. Glioblastoma multiforme cells progressively and irreversibly infiltrate the normal CNS, and tumors are highly resistant to radiotherapy and chemotherapy. The infiltration of the brain or spinal cord by malignant gliomas is pathologically evident by the juxtaposition of normal cellular elements with relatively isolated tumor cells at the periphery of the glioma in white matter tracts, around neurons, and under the pia (so-called Sherer’s structures).

Glioma cell interactions with the extracellular matrix facilitate their invasiveness and dispersal throughout the CNS. In contrast to most other tissues, the CNS extracellular matrix is organized by interactions of its components with hyaluronan (HA; refs. 1–3), a large, linear glycosaminoglycan that is especially concentrated in regions of high cell division and invasion. Moreover, HA expression and signaling are elevated in the matrices of many tumor types, including gliomas (4). Glioma cells produce matrix that is highly enriched in HA (5, 6), and these cells express high levels of the HA receptors CD44 and receptor for HA-mediated motility (7–9).

Numerous studies have shown that HA receptor interactions are important for glioma invasiveness (10–14). Specifically, we showed that antagonists of constitutive HA interactions strongly inhibit the invasiveness of several glioma cell lines in vitro (15). For example, small HA oligosaccharides (o-HA) that inhibit attachment of the intact multivalent HA polymer to its receptor CD44 reduce invasiveness by down-regulating key signaling receptor tyrosine kinases (RTK), including epidermal growth factor receptor (EGFR) and c-MET, which are important in glioma invasiveness (16, 17). o-HA down-regulate the activities of the phosphoinositide 3-kinase/Akt pathway (15, 18), which may mediate radioresistance (19). They also reduce chemoresistance and expression of drug efflux transporters that contribute to chemotherapy resistance (20, 21); these...
transporters are enriched in cancer stem cells (22). Therefore, HA antagonists, such as o-HA that target the signaling processes HA supports or initiates, may prove beneficial therapeutically.

Recent work has emphasized the potential role of cancer stem–like cells in malignancy and resistance to therapy in a variety of cancers. These cells have been variously named cancer stem cells, cancer progenitor cells, and tumor-initiating cells. They are highly malignant in that a very small number can rapidly generate a fully grown tumor when implanted in an animal host (23–25). They are also resistant to various therapeutic treatments and may be central to tumor recurrence (22). The exact nature of these tumor subpopulations is controversial, especially with respect to their precise relationship to stem cells (25), but the presence of highly malignant, therapy-resistant subpopulations within human tumors is reasonably well established. Expression of the HA receptor CD44 is frequently associated with these stem-like cells (23) but, although HA facilitates hematopoietic stem cell migration and homing (26, 27), very little is known about the relationship of HA to cancer stem cell or glioma progenitor cell behavior. Therefore, we have begun to investigate the relationship of HA-CD44 interaction to the properties of cancer progenitor cells. Because we are particularly interested in the role of HA in resistance to therapy, we have focused herein on its relationship to the ABC family drug transporter BCRP/ABCG2, because this transporter has been associated with drug resistance in cancer progenitor cells and is the molecular basis of progenitor cell enrichment within the so-called side population (SP) obtained by Hoechst dye exclusion in fluorescence-activated cell sorting (FACS) of cancer cells (28, 29).

Accordingly, we sought to determine if abrogating HA-CD44 interactions with o-HA effectively suppressed malignant properties of glioma and glioma progenitor cells in vitro and in a spinal cord model of invasive glioma that replicates invasive behaviors of human gliomas in the CNS. The data show that o-HA hold great promise for development as a novel biological therapy for the treatment of malignant CNS tumors.

Materials and Methods

Chemicals. Methotrexate was purchased from Tocris. Verapamil was provided by Dr. Lindsay Devane at the Medical University of South Carolina. All other reagents were purchased from Fisher Scientific, unless otherwise specified.

Cell culture. C6/lacZ7 rat glioma cells were obtained from the American Type Culture Collection. Cells were routinely cultured in DMEM supplemented with F12 (DMEM/F12, 50:50) with 10% fetal bovine serum and 1% penicillin/streptomycin. Expression of β-galactosidase was confirmed by immunostaining with β-galactosidase antibody (1:100; Cortex Biochem).

o-HA. Highly purified o-HA were fractionated from testicular hyaluronidase digests of HA polymer by tangential flow filtration, as described previously (30), and were donated by Anika Therapeutics, Inc. The average molecular weight of these oligomers was 2.5 × 10^3 (−3 to 10 disaccharide units). The oligomers were analyzed by high-performance liquid chromatography and capillary electrophoresis, and no contaminants were detected. Specific analyses for other glycosaminoglycans, protein, nucleic acids, and endotoxins were negative.

Flow cytometry. To identify and isolate SP cells in C6 glioblastoma cultures, cells were cultured in DMEM/F12 with 10% fetal bovine serum. After a well-established method for isolation of SP cells (31, 32), we labeled the cells at 37°C for 90 min with 2.5 μg/mL Hoechst 33342 dye (Molecular Probes, Invitrogen) and counterstained with 1 μg/mL propidium iodide to label dead cells. Cells were analyzed by FACS (MoFlo High Performance Cell Sorter by Dako Cytomation) using dual-wavelength analysis (blue, 424-444 nm; red, 675 nm) after excitation with 350-nm UV light. Propidium iodide–positive dead cells (≤15%) were excluded from the analysis. Cells were collected from the SP, which efflux Hoechst dye, and from the non-SP cells, which retain the dye. Gates were set based on inhibition of Hoechst dye efflux by the ABC transporter inhibitor verapamil.

Culture of SP and non-SP cells. Cells were maintained in serum-free medium containing N1 supplement (Sigma-Aldrich) and growth factors for neural stem cell survival. Media were supplemented with additional growth factors, such as platelet-derived growth factor, basic fibroblast growth factor (32), and epidermal growth factor (33), all obtained from BD Biosciences. Cells were sequentially transferred from 96-well plates to 24-well plates to 25 mm² flasks to 75 mm² flasks. SP cells were grown in suspension, forming sphere-like clusters even at low density. Upon exposure to 10% serum-containing medium, SP cells become adherent and took on a fibroblast-type morphology. For this reason, cells were maintained under serum-free conditions throughout subsequent experiments.

Morphology and demonstration of multipotency by immunostaining of cultured cells for neural, and glial-specific markers. The morphology of SP and non-SP cells was examined using phase contrast microscopy. The cells were cultured overnight in LabTEK II CC2 chamber slides. To show the neoglial multipotency of the SP, the expression of neuronal and glial markers in cultured cells was determined by immunocytochemistry. To induce differentiation, SP cells were cultured in the absence of growth factors on Matrigel (BD Biosciences)–coated slides for 7 d and then processed for immunostaining. The cells were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature, blocked with TBS containing 3% bovine serum albumin and 0.1% Triton-X100, and then stained with the following antibodies: anti–glial fibrillary acidic protein (GFAP; 1:200; DAKO), anti-III tubulin/Tuj1 (1:200; Sigma-Aldrich), anti–microtubule-associated protein 2 (1:500; Sigma-Aldrich), and anti-nestin (1:200; Chemicon). The primary antibodies were detected with fluorophore-conjugated secondary antibody (Alexa Fluor 488 and 555, 1:100, Molecular Probes, Invitrogen). The cells were counterstained with Hoechst 3342 dye (Molecular Probes, Invitrogen) to visualize nuclei. Immunostaining was done for BCRP expression using the same method, but with a BCRP-specific antibody (1:50; mouse monoclonal IgG2a clone BXP-21 from Kamiya Biomedical Company). This antibody does not cross-react with PgP (MDR1 gene product), MRPI, or MRP2, as shown by Kamiya Biomedical. Rabbit polyclonal CD133 antibody (1:50) was obtained from Abcam.

Western blot analysis. C6 cells were grown in six-well plates. Approximately, 10^5 cells were plated in DMEM/F12 in each well. After 48 h, o-HA (100 μg/mL in PBS) or PBS alone was added to the cells. After another 24 h, the cells were collected, lysed, and analyzed by SDS-PAGE and Western blotting using a BCRP-specific antibody (1:1,000; mouse monoclonal BXP21, Kamiya Biomed). Horseradish peroxidase–linked goat anti-mouse antibodies were purchased from Amersham Biosciences. Bands were revealed by Chemoluminescence Reagent Plus (PerkinElmer Life Sciences, Inc.), and protein sizes were estimated with size markers. Intensity of the bands was quantified by densitometry. Immunoreactive bands were quantified by densitometry and normalized to β-actin expression using a mouse monoclonal antibody (Ambion, Inc.).

RTKs and activation of downstream effector molecules (pAkt, pEGFR, and BCRP). C6 and the stem cell-like SP (C6SP) were analyzed by immunocytochemistry for BCRP expression, Akt activation (1:100; phosphorlated Akt 1/2/3 rabbit polyclonal; Santa Cruz Biotech), and EGFR activation (1:100; mouse monoclonal phospho-related EGFR; Upstate).
**Results**

**Effects of o-HA on pathogenesis of C6 gliomas in vivo** C6 glioma cells were reproducibly engrafted into the rat spinal cord and invaded in a rostrocaudal fashion. The engrafted C6 tumors shared many histologic features of invasive human gliomas, including invasion along white matter tracts, perineuronal satelliting, and subpial extension (Fig. 1A).

To determine whether o-HA has overt toxic effects, spinal cords without tumors were injected with 1 μg of o-HA in PBS or with PBS alone and examined 72 hours after o-HA administration. These cords did not show evidence of T cell or macrophage infiltration or microglial activation. There was a mild gliotic response after o-HA injection, but this was similar to that seen after injection with PBS alone. Rats injected with o-HA did not show weight loss, impaired mobility, or evidence of paralysis (data not shown).

The effects of o-HA on growth and invasion of C6 glioma cells were assessed by injecting rat spinal cords with lacZ-expressing C6 glioma cells (confirmed in vitro by immunostaining with β-galactosidase antibody with 99-100% of cells staining positive; data not shown) and allowing the cells to grow for 7 days. At this stage, tumors of ~2 mm in size had formed (Fig. 1B and D). These tumors were treated on day 7 with o-HA (100-1,000 ng in 1 μL PBS) or with PBS alone (1 μL) via injection into the epicenter of the tumor. In the PBS controls, additional tumor growth and infiltration along the spinal cord was evident by 14 days after engraftment (Fig. 1B and C). However, treatment with o-HA significantly decreased tumor growth (Fig. 1B-D), and tumors treated with o-HA showed little evidence of invasion beyond the site of engraftment, as detected by β-galactosidase immunohistochemistry (Fig. 1B). Results from various concentrations of o-HA are summarized on the graph (Fig. 1D).

In addition to analyzing the effects of o-HA on tumor growth, we assessed the effects of o-HA on cell proliferation, apoptosis, and signaling. We found that Ki67 immunostaining, a measure of cell proliferation, was almost totally inhibited in residual tumors after treatment with o-HA compared with PBS controls (Fig. 2). Furthermore, TdT-mediated dUTP nick-end labeling staining.
labeling staining, a measure of apoptosis, was increased in o-HA–treated tumors compared with PBS controls (Fig. 2). o-HA had no apparent effect on CD44 expression (data not shown), although it decreased activation and nuclear localization of Akt (Fig. 3) and reduced the number of BCRP-positive cells around vessels within the tumor (Fig. 4A). Interestingly, few BCRP-expressing cells seemed to be C6 glioma cells, as immunostaining of BCRP often did not correspond to β-galactosidase expression by the C6 cells (Fig. 4B). However, many BCRP+ cells coexpressed nestin (Fig. 4B), a marker of...
neural stem cells and endothelial precursors (35). Lack of staining for β-galactosidase and GFAP, a marker of activated astrocytes, showed that cells surrounding the vessels were not reactive astrocytes. Additionally, some cells surrounding the vessels were also positive for CD45, which suggests they may represent hematopoietic-derived cells (Fig. 4B).

**Isolation and characterization of C6SP cells.** Because BCRP+ cells were largely absent in vivo after treatment of C6 gliomas with o-HA, we isolated and characterized an SP from C6 glioma cells that express high levels of BCRP. The C6 cells were labeled with Hoechst dye, counterstained with propidium iodide to label dead cells, and then sorted by FACS. Cells with functional BCRP are able to efflux Hoechst dye. An SP of 0.74% was detected (Fig. 5A). This was reduced to 0.01% by treatment with verapamil, which inhibits ABC transporters, such as BCRP. Sorted cells were collected and expanded in serum-free neurosphere-inducing conditions, as previously described (32). The C6 total population was also analyzed by FACS for CD133, a marker for glioma progenitors, and yielded a subpopulation of 2% (data not shown). Sorted C6SP cells showed increased immunostaining for both CD133 and BCRP compared with the C6 non-SP (Fig. 5B). In the C6SP, BCRP seemed to be localized to the cell membrane, whereas in C6 non-SP, the staining pattern for BCRP indicated a cytoplasmic arrangement.

A previous report suggested that C6 glioma SP cells are multipotent (32). To confirm this, SP cells were grown in chamber slides for 7 days to promote differentiation of progenitor cells. After incubation, slides were stained with cell-specific markers for glia (GFAP), progenitor cells (nestin), and neurons (Tuj1 and microtubule-associated protein 2) and found to be positive for these markers (Fig. 5C). Also, as reported previously (32), C6SP were found to be highly tumorigenic, in that as few as 2,000 C6SP cells were able to grow and invade the rat spinal cord (Fig. 5D), whereas injection of 2,000 C6 non-SP failed to produce invasive gliomas (data not shown). Therefore, the C6SP cells isolated in this study are similar to SP cells isolated by others from the C6 glioma cell line (32).

**Effects of o-HA on properties of C6SP cells.** To assess the effects of o-HA on BCRP expression, the C6SP cells were cultured under serum-free conditions and treated with 100 μg/mL o-HA for 24 hours. After incubation, cell lysates were prepared, and immunoblots were done using BCRP-specific antibodies. C6SP expressed more BCRP than the C6 total population (Fig. 6A). Pretreatment of cells with 100 μg/mL o-HA for 1 hour reduced expression of BCRP in the C6SP cells. A similar decrease in BCRP was observed by immunofluorescence staining for BCRP (Fig. 6B). In addition, C6SP expressed more activated Akt (pAkt) than the C6 total population (C6) and pretreatment with o-HA for 1 hour reduced activation of Akt and EGFR in the C6SP cells (Fig. 6B). To assess the effect of o-HA treatment on the function of BCRP, we tested C6 and C6SP cells for resistance to methotrexate, a drug that is effluxed by BCRP, as well as other ABC transporters. As shown in Fig. 6C (left), C6SP cells were 1,000-fold more resistant to methotrexate than C6 parental cells. Survival of C6SP glioma cells was decreased by treating cells with o-HA (100 μg/mL for 24 hours; Fig. 6C, right).

Finally, we showed that treatment of highly resistant C6SP tumors in vivo with o-HA (5 μg single intratumoral injection) significantly reduced tumor size compared with PBS-treated animals (Fig. 6D). In tumors generated from C6SP cells, some
BCRP-positive cells that were also positive for β-galactosidase persisted (Fig. 4A). However, the majority of the β-galactosidase–positive tumor cells were weakly positive for BCRP, suggesting that the SP cells had undergone differentiation in the tumor.

**Discussion**

We have shown that the growth of malignant gliomas is suppressed by antagonizing constitutive HA-CD44 interactions with o-HA. o-HA promote apoptosis in glioma cells, whereas abrogating recruitment of host-derived nestin+/CD45+/BCRP+ progenitor-like cells. Our data also show that o-HA suppress Akt and EGFR activation, as well as expression of BCRP, a marker of drug-resistant glioma progenitor cells.

Various animal models of malignant gliomas have been used, ranging from engraftment of rodent and human cell lines into the CNS to use of orthotopic human glioblastoma xenografts and genetic mouse models, among others. Glioma cells engrafted into the rat spinal cord, unlike those engrafted into the brain, show patterns of dispersal that strongly resemble malignant human gliomas, including invasion of Sherer’s structures: white matter, subpial, and HA-rich perineuronal nets. Although spinal cord gliomas account for <10% of CNS gliomas, our animal model provides an effective way to focus on the effects of HA antagonism on malignant glioma growth and...
Fig. 5. Isolation and characterization of cultured C6SP cells. A. C6 glioma cells were labeled with Hoechst dye and analyzed by flow cytometry, as described in Materials and Methods. An SP of progenitor cells (0.74%) was isolated from C6 rat glioma cells by Hoechst dye efflux and FACS analysis. Treatment with verapamil (50 μmol/L) eliminates the SP. B. C6SP and non-SP cells were collected by FACS and cultured in serum-free conditions on chamber slides. Cells were immunostained for BCRP expression (green) and CD133 (red) in C6SP (spheres and single cells) and C6 non-SP (cells that retain Hoechst dye). C6SP grew as spheres and some attached single cells, whereas C6 non-SP did not form spheres and only grew as attached cells. Expression of BCRP (green) and CD133 (red) was greater in C6SP spheres and C6SP single cells compared with C6 non-SP, which had a very low level of detectable BCRP or CD133. Bar, 50 μm. C. C6SP was cultured on Matrigel-coated slides for 7 d in serum-free conditions to induce cell differentiation. Multipotency of C6SP is shown by expression of neuronal markers Tuj1 (green) and microtubule-associated protein 2 (red), as well as GFAP and stem/progenitor marker (nestin). D, 2,000 C6SP cells were engrafted into rat spinal cord and allowed to grow for 7 d, after which tissue was collected and immunostained for β-galactosidase to detect tumor cells and nestin, which stains both C6 tumor cells and progenitor cells. C6SP cells (red, β-galactosidase) and other nestin-positive cells (green) were noted at a distance from the epicenter of their engraftment (arrows). Tumor cells (red, β-galactosidase) that also express nestin are seen as yellow. Some nestin-positive cells were β-galactosidase negative, indicating they were not tumor cells but probably were neural or endothelial progenitor cells recruited to the tumor site. Nuclei are labeled with Hoechst dye (blue). Individual images were merged in Adobe Photoshop as a montage.
invasiveness. In this model, we have now shown consistent engraftment, growth, and invasion of C6 glioma cells, as well as highly tumorigenic SP progenitor cells isolated from the C6 cell line (C6SP).

Many studies have focused on signal transduction pathways and specific RTKs as targets for development of novel therapeutic agents. Although targeted RTK inhibitors have been developed for the treatment of solid tumors, including gliomas (36), it seems clear that multiple pathways will need to be targeted to maximally suppress glioma cell proliferation, especially in subpopulations of glioma progenitor cells enriched for BCRP and other drug transporters that efflux RTK inhibitors (22, 37–40). Because disrupting HA interactions by treatment of tumor cells with o-HA has been shown to inhibit activation of multiple RTKs (17) and expression of ABC family drug transporters (21), we tested their efficacy in glioma cells and BCRP-enriched glioma progenitor cells. As expected, we found that the o-HA inhibited activation of EGFR and Akt, effects which are most likely responsible for their suppression of glioma growth and promotion of apoptosis in vivo. In addition, o-HA down-regulated BCRP expression in drug-resistant and multipotent glioma progenitor cells. Thus, our results indicate that HA interactions are crucial to maintaining both tumorigenicity and stem cell–like features in cancer cells.

Evidence has been published showing that BCRP localization (and therefore function) is regulated by Akt activation (41). As expected from our previous studies demonstrating that o-HA down-regulates the activity of the phosphoinositide 3-kinase/Akt pathway (15, 18) and consequently reduces expression of drug efflux transporters (21), we found here that o-HA treatment of BCRP-enriched C6 glioma cells inhibits activation of Akt and that decreased Akt activation is associated with decreased BCRP expression (Fig. 6). Thus, we speculate that Akt activation may be involved in regulation of BCRP in glioma progenitor cells and that HA regulates these activities, which may otherwise confer resistance of glioma stem cell–like populations to chemotherapy. Further studies will be needed to confirm these findings and delineate the pathways involved.
Defining these HA/CD44-dependent signaling pathways in radiation-resistant and drug-resistant glioma stem cell–like populations is important because HA antagonism may amplify the benefits of current therapies. o-HA may enhance cytotoxicity of various chemotherapy agents and RTK inhibitors by decreasing ABC transporter–mediated cellular efflux. Because Akt activation is linked to radiosensitivity in rodent and human glioblastomas (42, 43) and because Akt activation is suppressed by o-HA in vitro and after a single injection of o-HA treatment of gliomas in vivo, we believe o-HA may also potentiate the therapeutic effects of radiation.

One of our most interesting observations from studying gliomas in vivo is their infiltration by nonglioma (β-galactosidase negative) BCRP+ cells, concentrated primarily around blood vessels within the tumor. At least some of these cells coexpress nestin or CD45 and thus may be progenitor cells. Their predilection for vessels contained in the gliomas suggest they could represent pericytes or endothelial progenitors (44). Glioma-secreted SDF-1 may function through a CXCR4/extracellular signal-regulated kinase/Erk-linked signaling pathway to modulate migration of nestin + neural progenitor cells (46). Glioma-synthesized factors, such as SDF-1, vascular endothelial growth factor, glioma-secreted mediators of vasculogenesis (27). In the developing spinal cord, SDF-1 may function through a CXCR4/extracellular signal-regulated kinase/Erk-linked signaling pathway to modulate migration of nestin + neural progenitor cells (46).

Gliomasynthesized factors, such as SDF-1, vascular endothelial growth factor, and other chemokines factors, probably attract CNS-derived or marrow-derived cells toward the glioma. Thus, glioma-derived HA not only increases key cellular signaling pathways that mediate malignant behaviors in autocrine fashion, but also likely enhances recruitment of neural and extraneuronal cellular elements that promote tumor growth and invasion in the CNS.

Our findings have implications for therapeutic development and refinement of HA antagonism with HA oligomers. We have shown that glioma cell proliferation, invasiveness, and treatment resistance are influenced by the autocrine action of HA-CD44 interaction on key RTKs, such as EGFR and c-MET, on downstream cell survival activities, such as Akt, and on the ABC family of drug efflux transporters. Our data also show that BCRP-rich C6SP are multipotent, invasive in vitro and in vivo, and highly resistant, due in part to BCRP efflux of chemotherapeutic drugs. Importantly, from a therapeutic perspective, o-HA inhibited activation of EGFR and Akt and expression of BCRP in C6SP. In addition, treatment of cells with o-HA decreased the in vitro survival of C6SP after exposure to methotrexate. Taken together, our findings suggest that HA antagonism may effectively abrogate activation of multiple signaling pathways in glioma cells and in subpopulations of glioma progenitors, which may well account for high rates of tumor recurrence after radiation therapy and chemotherapy.

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