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The Combined Influence of Hydrogel Stiffness and Matrix-Bound Hyaluronic Acid Content on Glioblastoma Invasion

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Abstract

Glioblastoma (GBM) is the most common and lethal form of brain cancer. Its high mortality is associated with its aggressive invasion throughout the brain. The heterogeneity of stiffness and hyaluronic acid (HA) content within the brain makes it difficult to study invasion in vivo. A dextran-bead assay is employed to quantify GBM invasion within HA-functionalized gelatin hydrogels. Using a library of stiffness-matched hydrogels with variable levels of matrix-bound HA, it is reported that U251 GBM invasion is enhanced in softer hydrogels but reduced in the presence of matrix-bound HA. Inhibiting HA-CD44 interactions reduces invasion, even in hydrogels lacking matrix-bound HA. Analysis of HA biosynthesis suggests that GBM cells compensate for a lack of matrix-bound HA by producing soluble HA to stimulate invasion. Together, a robust method is showed to quantify GBM invasion over long culture times to reveal the coordinated effect of matrix stiffness, immobilized HA, and compensatory HA production on GBM invasion.

Graphical abstract

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Supporting Information: Supporting Information is available from the Wiley Online Library or from the author.

Keywords

bioengineering; glioblastoma; hydrogel; invasion; stiffness

1. Introduction

Glioblastoma (GBM) is the most common and lethal form of brain cancer.^[1] It is a WHO grade IV astrocytoma, is marked by rapid, diffuse infiltration throughout the brain and exhibits low survival rates (15 months median; <5% after 3 years).^[1–3] Conventional clinical approaches rely on surgically debulking the tumor mass followed by chemo and radiotherapy. While introduction of temozolomide as a current standard of care has lengthened patient survival time, continued progress is necessary to address this devastating disease.^[4] A primary contributing factor to this poor response is the means by which GBM spreads. While many cancers display metastatic spreading to secondary tissue sites around the body via conserved processes,^[5] GBM rarely metastasizes outside the brain.^[6] Instead, GBM relies on diffuse spreading throughout the brain, with GBM cells invading along structural features (e.g., white matter tracts, blood vessels) within the brain.^[7] Complicating efforts, GBM often presents clinically at an advanced stage after extensive invasive spreading, resulting in poorly defined tumor margins. And unlike surgical intervention for many cancers where wide margins or total tissue resection are possible, GBM surgical margins are sharply defined. As a result, it is essential to consider how the tissue environment in the tumor and surrounding parenchyma impact invasion. While GBM tumors contain increased fibrillar matrix proteins (fibronectin, collagen),^[8] hyaluronic acid (HA) is the main extracellular matrix component of brain tissue also believed to alter glioblastoma invasive phenotype.^[9–12] As a result, the main cell surface receptor for HA, CD44 is thought to play an important role in altering GBM invasion via direct CD44–HA interactions.^[10]

A number of studies have begun to explore the use of in vitro platforms to quantify glioblastoma invasion.^[13–17] Many studies examining invasive phenotype have relied on 2D

substrates and tracing individual cell motility over time.^[18,19] While these substrates allow for ease of time-lapse imaging, there is evidence suggesting that the lack of 3D cell–matrix interactions significantly reduce the capacity to predict invasion within a 3D tumor.^[3,20–23] Semi-3D assays using Transwell membranes^[24,25] and microfluidic channels^[26] offer additional insight regarding the effects of confinement and biomolecular cues on cell motility. And Matrigel, a basement membrane preparation from murine sarcoma, has been used to show chemoattractants such as soluble HA may promote cell invasiveness.^[27,28] However, lack of control over gelation and final network structure^[29] as well as batch variability has made it difficult to quantitatively examine cell–matrix interactions in the context of GBM invasion. And while in vivo efforts add important insight,^[30] they involve long experimental time points and limited opportunities for in situ analysis of cell behavior.

Hydrogel biomaterials have increasingly been used as in vitro culture platforms to explore the behavior of glioblastoma cells in a matrix inspired environment.^[31] Synthetic polymers such as poly(ethylene-glycol) or polyacrylamide have been widely used to examine the effect of matrix stiffness on GBM cell morphology, proliferation, and migratory behavior as well as on the delivery of HA or select matrix metalloproteinases to alter cell response.^[32,33] Naturally derived matrix (e.g., collagen or hyaluronic acid) hydrogels have recently been described as culture platforms for GBM, though reduced control over degree of crosslinking and degradation sites remain critical challenges.^[14,34–36] Our lab has described a methacrylamide-functionalized gelatin (GelMA) hydrogel able to selectively incorporate matrix-immobilized hyaluronic acid.^[37–39] Gelatin is a denatured form of collagen which contains cell binding Arg-Gly-Asp (RGD) sites as well as MMP-sensitive degradation sites, making it attractive for generating a series of in vitro culture environments with tuned biophysical (e.g., stiffness) properties.^[39] The methacrylamide sites on the gelatin backbone also provide the opportunity to covalently immobilize hyaluronic acid, a primary component of brain matrix reported to alter glioma behavior,^[40] within the hydrogel. Our previous work has used these hydrogels to show that matrix-immobilized HA content is a critical regulator of the expression of malignancy and matrix-remodeling associated genes in GBM cells.^[38]

The primary goal of this manuscript is to explore the effect of key matrix biophysical parameters, stiffness, and covalently immobilized HA content, on the invasive phenotype of GBM cells within a fully 3D hydrogel. While quantitative analysis of 3D cell invasion is typically accomplished for single cells via cell-tracking algorithms,^[41,42] such studies can be limited by the length of tracking time. Here, we were inspired by scratch test studies typically performed on 2D substrates,^[43,44] where the scratch leaves behind a well-defined starting point from which to measure cell movement. We employ a bead assay where GBM cells are first seeded onto Dextran beads coated in denatured collagen; cell-coated beads are then encapsulated within the hydrogel, with the bead surface providing an accurate starting point for GBM invasion into the surrounding hydrogel. Research groups studying in vitro vascular network formation and the kinetics of melanoma invasion have previously reported the use of commercially available beads for cell-bead-hydrogel cocultures, though typically with less defined hydrogel carriers (e.g., fibrin, collagen).^[45,46] While those approaches typically employ total spread area as a metric of cell spreading, we quantify cell invasion distance of each cell from the surface of the bead over extended culture (up to 7 d) times. The nature of the assay removes the need for intense fluorescence exposure often required to

measure invasion metrics in 3D biomaterials.^[41] And while GBM cell invasion has previously been reported using neurospheres encapsulated within a hydrogel network,^[47,48] quantification of cell spreading from neurospheres remains difficult due to an inability to rigorously identify the initial starting position of the neurospheres.^[27] As a result, we report the effect of both matrix stiffness and incorporation of matrix-bound HA on the metabolic activity and invasive phenotype of GBM cells. We further explore potential underlying mechanisms behind changes in GBM invasion by examining the impact of CD44–HA interactions on gene expression profiles, production of HA biosynthesis and degradation proteins, and secretion of soluble HA.

2. Experimental Section

2.1. Preparation of Methacrylated Gelatin and Hyaluronic Hydrogels

Methacrylated gelatin and methacrylated hyaluronic acid (HAMA) precursors were fabricated as previously described.^[38] Briefly, gelatin (Type A, 300 bloom from porcine skin, Sigma-Aldrich, St. Louis, MA) was dissolved in phosphate buffered saline (PBS; Lonza, Basel, Switzerland) at 60 °C. Methacrylic anhydride (MA) was then added dropwise into the solution, and the reaction proceeded for an hour. The methacrylated gelatin product was dialyzed with 12 000–14 000 molecular weight (MW) cutoff dialysis tubing (Fisher, Pittsburgh, PA) for one week against 40 °C deionized water (DI water), and was then isolated via lyophilization. HAMA was synthesized by adding methacrylic anhydride dropwise into a 4 °C solution of HA sodium salt (60 kDa, Lifecore Biomedical, Chaska, MN) in DI water. The pH was adjusted to 8 via 5 N NaOH, and the reaction was allowed to proceed overnight at 4 °C. The product was then purified via similar dialysis and lyophilization steps as GelMA. The degree of functionalization of both GelMA and HAMA was determined by ¹H NMR as 50% (±2%) degree of MA functionalization.^[38]

Hydrogels were then prepared from mixtures of GelMA and HAMA in PBS at a total concentration (GelMA + HAMA) of 4 wt% or 5 wt%. To examine the effect of HAMA within the GelMA matrix, the ratio of HAMA:GelMA (0:100, 10:90, 15:85 HAMA:GelMA w/w) was adjusted while maintaining the overall wt% of each mixture (4, 5 wt%). Cells were added to the prepolymer solution immediately prior to being placed into Teflon molds (0.2 mm thick, 5 mm radius). The mixture was then photopolymerized under UV light (AccuCure LED 365 nm, Intensity 7.1 mW cm⁻² for 30 s) in the presence of a lithium acylphosphinate (LAP) photoinitiator (PI) as previously described.^[49] The amount of LAP photoinitiator was adjusted to allow fabrication of HA containing versus GelMA only constructs with identical mechanical properties (0.1 wt% LAP for GelMA hydrogels; 0.02 wt% LAP for GelMA/HAMA hydrogels). Cells containing hydrogels were subsequently maintained in culture media at 37 °C and 5% CO₂ for all experiments.

2.2. Characterization of Elastic and Diffusive Properties of Gelatin Hydrogels

The compressive modulus of each hydrogel variant was measured using a mechanical testing machine (Eden Prairie, MN). Briefly, hydrogels were compressed at the rate of 0.1 mm min⁻¹, with the Young's modulus obtained from the linear region of the stress–strain curve (0–10% strain) as previously described.^[38] In addition, the diffusivity of each hydrogel

variant was determined via fluorescence recovery after photobleaching (FRAP) experiment using a confocal microscope (Zeiss LSM710 Multiphoton Confocal Microscope, Germany) and a 40 kDa FITC-dextran probe.^[50] Briefly, acellular hydrogels were incubated in fluorescein isothiocyanate (FITC) probe containing PBS (1 $\mu\text{g mL}^{-1}$) overnight. A 488 nm laser was then used to photobleach a circular spot (radius 23.6 μm) within the hydrogel. The fluorescence intensity (I) within that region during recovery was fit to

$$I=A_1(1-e^{-\tau t}) \quad (1)$$

in order to determine the time constant (τ) of recovery. The diffusion coefficient (D) of each hydrogel was subsequently calculated as described by Reits et al.^[50]

$$D=\omega^2/4t^{1/2} \quad (2)$$

$$t^{1/2}=\ln(0.5/-\tau) \quad (3)$$

2.3. Cell Culture

U251MG cells (a gift from Dr. Jann Sarkaria, Mayo Clinic, MN), known to be an invasive GBM cell line,^[51–53] were cultured in Dulbecco's modified eagle medium (Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (Lonza, Basel, Switzerland). Cells were incubated at 37 °C in a 5% CO₂ environment and passaged when reaching confluence. For analysis of cell metabolic health, protein expression and gene expression, U251MG cells were homogeneously mixed with the GelMA/HAMA solution at a density of 4.0×10^6 cells mL^{-1} . Cell-seeded hydrogels were incubated in cell culture medium at 37 °C, 5% CO₂ in low adhesion well plates containing standard culture media.

2.4. Time-Lapse Analysis of Cell Invasion

To measure relative cell motion in the fully 3D hydrogel environment, a 3D assay was used to examine invasion of populations of cells away from a defined starting position. Here, collagen-coated dextran beads ($\approx 200 \mu\text{m}$ dia.; GE Life Sciences, Pittsburgh, PA) were hydrated in PBS, washed overnight, then sterilized via autoclave before being resuspended in fresh culture media. Dextran beads (10 000) were then incubated in a 5 mL solution of U251MG cells (4×10^6 cells) suspension under light agitation (one minute, every thirty minutes) over a period of 4 h to facilitate cell attachment to the beads. Cell-coated beads were subsequently incubated overnight in cell culture media before being mixed with the hydrogel precursor suspension (≈ 10 beads/25 μL hydrogel solution) and encapsulated into the final hydrogel matrix.

Cell invasion into the hydrogels away from the bead surface was quantified after 3 and 7 days in culture. Briefly, bead-laden hydrogels were fixed using Formalin solution (Sigma) and then were washed in PBS washing. The nuclei of individual cells within the hydrogel matrix were stained with Hoechst 33342 (ThermoFisher, Waltham, MA) at a concentration of $4 \mu\text{g mL}^{-1}$. A series of images were acquired at different focal planes around individual beads using a Leica DMI 400B fluorescence microscope (Leica, Germany); analysis of cell position relative to the bead surface was then quantified via Image J (Analyze Particle function) for all image planes surrounding each bead after they were collapsed into a single image. Invasion distance was calculated for each cell as the linear distance from the bead surface to the center of each nuclei. Alternatively, some hydrogel samples were stained for both nucleus (Hoechst 33342) and actin cytoskeleton (Phalloidin, ThermoFisher) in order to generate 3D image stacks showing cell position and morphology (LSM 710 Multiphoton Confocal Microscope, Zeiss).

2.5. Analysis of Cell Metabolic Activity

Total metabolic activity of cell-seeded hydrogels was analyzed using a dimethylthiazol-diphenyltetrazolium bromide (MTT) assay (Molecular Probes, Waltham MA) via previously described methods.^[37] Analysis was performed on cells encapsulated within the hydrogel without dextran beads. Briefly, total metabolic activity was measured immediately following hydrogel encapsulation (day 0) and then subsequently at days 3 and 7 of hydrogel culture. Briefly, at each time point the culture media surrounding each hydrogel sample was replaced with MTT-containing media for 4 h then dimethyl sulfoxide (Sigma) overnight. Metabolic activity of each cell-seeded hydrogels was measured via absorbance using a microplate reader at 540 nm (Synergy HT, Biotek, Vermont, VT), with data normalized to day 0 samples (immediately after seeding).

2.6. Hyaluronic Acid Secretion

Total soluble HA produced by cell-seeded hydrogels over 7 days in culture was quantified from media samples via enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, MN) following manufacturer's instructions. The low (15-40 kDa), medium (75-350 kDa), and high (>950 kDa) molecular weight forms of HA are all detected by this assay. Samples were analyzed via a microplate reader (Synergy HT, Biotek), with HA secreted per cell calculated at day 7 from the initial number of seeded cells at day 0 and the relative change in metabolic activity per construct between days 0 and 7.

2.7. RNA Isolation and Gene Expression

Gene expression of hyaluronic synthase (HAS-1, HAS-2, and HAS-3) and hyaluronidase (HAdase-1, HAdase-2) of cells encapsulated within the hydrogel was determined by real-time Polymerase chain reaction (PCR) via previously described methods.^[37] RNA was extracted from cell containing hydrogels via the RNeasy Plant Mini kit (Qiagen, Valencia, CA). Isolated RNA was then reverse transcribed to cDNA in a Bio-Rad (Hercules, CA) S1000 thermal cycler using the QuantiTect reverse transcription kit (Qiagen). Real-time PCR was then performed in triplicates using QuantiTect SYBR Green PCR kit (Qiagen) with an Applied Biosystems 7900HT fast real-time PCR system (Carlsbad). Primers were synthesized by integrated DNA technologies (Table 1) using sequences derived from

literature.^[32] Results were normalized against cells seeded into the hydrogel (≈ 1 h) but then isolated immediately thereafter (day 0).

2.8. Protein Isolation and Western Blotting

Protein isolation and Western blotting procedures were accomplished as previously described.^[54] Briefly, proteins were extracted from cell containing hydrogels (hydrogels without beads) by immersing the samples in 0 °C RIPA buffer. Total protein concentration in the lysates was determined by BCA assay (Bio-Rad). Lysates were then mixed with 4 $\times$ Laemmli sample buffer (Bio-Rad), then loaded (5 μ g protein in 15 μ L per lane) onto polyacrylamide gels (Bio-Rad). Gel electrophoresis was performed at 150 V, with proteins then transferred onto nitrocellulose membranes using Trans-Blot SD (Bio-Rad). Membranes were blocked in 5 wt% nonfat milk in Tris-buffered saline then incubated with primary antibodies (Anti-HAS3 antibody, Anti-HYAL1 antibody, Abcam, UK; β -actin, Cell Signaling, Danvers, MA) at 4 °C overnight. Membranes were subsequently washed, then incubated with a secondary antibody (Anti-rabbit IgG HRP-linked, Cell Signaling) for at least 2 h at room temperature. Imaging signal was visualized using an Image Quant LAS 4010 (GE Healthcare), with band intensities quantified using ImageJ and normalized to β -actin expression.

2.9. Inhibition of CD44-Hyaluronic Acid Interactions

In order to explore the effect of CD44-mediated adhesion to hydrogel bound hyaluronic acid, experiments were repeated with media containing anti-CD44 antibody (1 μ g mL⁻¹, Calbiochem, San Diego, CA). Samples were collected as previously described to perform both metabolic activity (MTT) and invasion assays.

2.10. Statistics

All statistical analysis were performed using one-way analysis of variance (ANOVA) followed by Tukey-HSD post-hoc tests. A minimum of $n = 3$ (modulus, diffusivity), $n = 4$ (cell invasion), and $n = 3$ (MTT, ELISA, PCR, Western) samples were used for all assays. Statistical significance was set at $p < 0.05$. Error is reported as the standard error of the mean unless otherwise stated.

3. Results and Discussion

Unlike many other cancers that metastasize to a secondary tissue, GBM invades and infiltrates throughout the brain but rarely metastasizes outside.^[6] Many studies have sought to understand the mechanisms of GBM invasion and elucidate the external cues that trigger and promote invasive potential. Recent efforts exploring invasion using a 3D matrix often utilize spheroids, providing a model that includes a fully 3D environment and starting cell mass;^[31,55] however, the lack of a defined starting point makes it difficult to assess cell invasion over long culture times and after significant cell proliferation and invasion. Further, the large data sets typically obtained via continuous time-lapse fluorescent tracking make the analysis convoluted and time consuming. Here, the Dextran beads provide an easy to quantify starting location of all cells and allow more accurate tracing of cell invasion for nonradially symmetrical cell distributions. Applying this system, we describe the effect of

hydrogel biophysical (stiffness) and biomolecular (HA content) cues on invasive phenotype of a U251 GBM cell line previously used to explore GBM motility on 2D substrates.^[51]

3.1. Biophysical Properties of a Family of GelMA and GelMA-HAMA Hydrogels

The biophysical properties of the native brain and tumor mass can vary widely over the range of Pa to low kPa.^[56] Inspired by these changes, we explored the effect of matrix biophysical properties on GBM invasion for a subset of GelMA hydrogels created with low-kPa range mechanical properties. We generated two families of low (4 wt%) and high (5 wt%) density GelMA hydrogels, each containing three variants with increasing amounts of incorporated hyaluronic acid (0%, 10%, 15% w/w). The fraction of incorporated matrix-bound HAMA content was set based on our previous work^[38] showing the impact of HA on GBM cell behavior. To examine the effect of HAMA content in a manner that did not couple with hydrogel stiffness, we adjusted the PI content (0.1 wt% for GelMA only groups, 0.02 wt% for GelMA-HAMA groups) to maintain same modulus range within the same wt% groups (Figure 1A). Hydrogels fabricated at 5 wt% (12.8 ± 0.5 kPa) were significantly ($p < 0.05$) stiffer than 4 wt% (8.8 ± 0.4 kPa) hydrogels for all amounts of incorporated HA, with no significant difference as a function of HA incorporation. The diffusion coefficient for each variant was subsequently determined via FRAP using a 40 kDa FITC-dextran probe, finding that while matrix elasticity was defined by overall wt% (4 vs 5), diffusivity was dictated by HA content (0%, 10%, 15% w/w). Notably, while the diffusivity of hydrogels for a given wt% increased with increasing HA content, there was no significant effect of overall hydrogel wt% on hydrogel diffusivity (Figure 1B).

3.2. Cell Metabolic Activity and Invasive Spreading as a Function of Hydrogel Biophysical Properties

Hyaluronic acid is a main component of brain extracellular matrix and is believed to be strongly associated with many phases of tumor growth.^[57,58] Previous studies have explored the influence of HA molecular weight on cell response; while high-MW HA is usually associated with antiangiogenic and anti-inflammatory processes and low-MW HA more associated with proinflammatory and proangiogenic responses, boundaries remain vague.^[57,59] HA synthesis is driven by hyaluronan synthase (HAS), three variants (HAS-1, HAS-2, HAS-3) are responsible for different HA size ranges. HA degradation is driven by hyaluronidase (Hyal-1, Hyal-2), with hyaluronidase expression previously linked to GBM invasiveness.^[57] Recently, a range of efforts have begun to explore the use of HA-based hydrogels to examine GBM bioactivity.^[13,60] Here, we employ a HA-decorated GelMA hydrogel as a means to explore the potential balance between tumor-inspired fibrillar proteins and parenchyma-inspired HA on GBM invasion.^[61] The range of elastic moduli and HA content explored here were informed by native properties of GBM tumors in vivo^[62,63] as well as previous studies in our lab looking at the activation of GBM cells as a function of GelMA mechanical properties and HA content.^[37,61] While U251-coated beads are readily viewable via confocal microscopy, time-lapse brightfield provides sufficient resolution to trace GBM cell invasion into the hydrogel network away from the bead surface over many days (Figure 2A,B).

The metabolic activity of U251 cells increased significantly ($p < 0.05$) with culture time for all hydrogel variants, with no significant difference in metabolic activity between groups (Figure 2C,D). Not surprisingly, cell invasion increased significantly ($p < 0.05$) between days 3 and 7 for all groups. However, matrix biophysical properties significantly influenced the distance U251 cells invaded through the hydrogel network away from the dextran bead surface (Figure 2E,F). Cell invasion was significantly ($p < 0.05$) greater in the 4 wt% hydrogel variants. However for the series of 4 wt% hydrogels, mean invasion distance was significantly ($p < 0.05$) higher in hydrogels lacking matrix immobilized HA than those containing HA. And while not significant, the same trend was observed for the higher density 5 wt% hydrogels. This effect was more strongly observed when considering the most highly invasive subfraction of cells. Quantifying the invasive distance for the furthest spread 10% of cells in each group, we again note significant increase in invasive distance with time (day 7 greater than day 3), wt% (4 wt% greater invasion than 5 wt%), and HA content, with significantly increased invasion observed in HA-free variants (Figure S1, Supporting Information). Measures of metabolic activity of these cells showed no significant difference in metabolic health with the inclusion of HA, suggesting the decrease in invasion was not due to changes in proliferation or metabolic activity. While CD44 has also been reported to be a potential cancer stem cell marker,^[64] the U251 cell line used in this study did not show an appreciable stem fraction during culture (not shown). So while ongoing efforts in our lab are examining the relative invasion potential of GBM stem cells, results here likely do not include the coupled complexity of stem cell–HA interactions.

3.3. Inhibition of CD44 Resulted in Decreasing Cell Invasion

Based on the observation that cell invasion was reduced with increasing matrix-bound HA and to better understand the role matrix-bound HA plays in GBM invasion, we subsequently investigated potential pathways that could be responsible for this response. Given the role of CD44 as the principle receptor of HA as well as its recent potential as a target therapy for cancer applications,^[65,66] as well as its role in cell-attachment and invasion,^[11,67] we examined whether the invasion behavior of U251 was altered by blocking CD44–HA interaction by adding an antibody that specifically recognizes and reacts with CD44 receptor. For these experiments, we concentrated primarily on the series of hydrogels (4 wt %; 0, 10, 15% w/w HA) that showed the greatest invasion, though comparison was also made to the 5 wt%, 0% w/w HA hydrogel as well. We did not observe any changes in cell shape with the addition of anti-CD44 antibody. Not surprisingly, GBM cell invasion was significantly reduced in HA-decorated hydrogels when CD44–HA interactions were inhibited. Surprisingly, GBM cell invasion was also reduced in GelMA-only hydrogels with no matrix-bound HA when CD44–HA interactions were inhibited (Figure 3A,B). The effect was stronger at day 3 as opposed to day 7. These differences on invasion distance do not appear to be related to changes in cell proliferation, as similar metabolic activity trends were observed for the cells both without (Figure 3C) and with (Figure 3D) the inclusion of anti-CD44. We observed no significant differences between metabolic activity of matrix groups with and without anti-CD44 except for the 4 wt% GelMA hydrogel group ($p < 0.01$). However, the persistence of the observed difference in migration across all groups suggests that the reduced invasion distance with anti-CD44 treatment is more tied to CD44 inhibition than proliferation.

We hypothesized that glioblastoma cells may secrete soluble HA as a means to compensate for the lack of matrix-bound HA in the hydrogel network. As a result, we subsequently quantified total soluble HA within the culture media through day 7 via ELISA, with results normalized versus the total number of cells in the hydrogels at day 7 (Figure 4). Interestingly, GBM cells cultured in GelMA hydrogels lacking matrix-bound HA produced significantly ($p < 0.01$) greater amounts of soluble HA than cells in GelMA hydrogels with matrix-bound HA. These results suggest that U251 cells may compensate for the lack of HA in the matrix by producing soluble HA that can affect invasion processes. Interestingly, recent results in the literature by Kim and Kumar suggested that CD44-based signaling is intrinsically mechanosensitive, suggesting that the mode of presentation of HA (soluble, matrix-bound) may significantly alter invasive phenotype.^[67] In the native brain environment, both matrix-bound HA and soluble HA are present,^[68,69] suggesting the need for future experiments looking at the connection between matrix-bound versus soluble HA, activated signaling pathways, and resultant invasive phenotype. While we report U251 cell line invasion was reduced in the presence of matrix-bound HA and increased with endogenous production of soluble HA, this finding is separate from biphasic relationships often observed for adhesion-based interactions for other 2D and 3D biomaterial systems.^[70] While examining narrower ranges of HA content (between 0% and 10% w/w) may be useful to resolve potential biphasic interactions, results described here are suggestive that the balance of cell-produced soluble and matrix-bound HA may be an important consideration in the context of GBM invasion within the brain.

3.4. GBM Cells Are Capable of Producing Hyaluronidase and Hyaluronic Synthase

We further examined gene expression and protein synthesis profiles for HA remodeling associated targets, examining expression profiles for subtypes of both hyaluronidases, the protease responsible for HA-matrix degradation during remodeling,^[71] and hyaluronic synthase, responsible for producing soluble HA and linked to invasion.^[32,67] Here, we chose to compare gene expression and protein synthesis profiles for a subset of 4 wt% hydrogels that either contained (15% w/w HA) or did not contain (0% w/w HA) HA, screening for three HAS genes (HAS-1, HAS-2, HAS-3) and two HAdase genes (Hyal-1, Hyal-2). The results showed that U251 has active HAS and HAdase gene expression, but revealed no significant trend as a function of matrix environment at the time-points explored (Figure 5). While expression levels were in most cases elevated in HA-containing hydrogels versus GelMA only variants, the results were not significant. Further, addition of anti-CD44 did not appear to significantly alter any gene expression profiles. We subsequently examined protein expression for Hyal and HAS subtypes that showed the highest gene expression variations and that are responsible for large molecular weight HA (HAS-3, Hyal-1) via Western blot (Figure 6). Here, we confirmed that U251 cells produce quantifiable levels of HAdase (Hyal-1) to degrade matrix-bound HA, suggesting differences in invasion distance as a result of matrix-bound HA was not due to the inability to degrade the matrix. Further, we confirmed that U251 cells secrete HAS-3, but found no specific effect of matrix-bound HA or CD44 inhibition on protein secretion levels.

3.5. Future Extensions of This Work

Recent work examining the failure of molecular subtype therapies in cancer have described the potential that compensatory signaling pathways capable of bypassing canonical pathways may be responsible for the clinical failure of these therapies. While this work has largely focused on intracellular processes and extracellular proteome changes,^[72–74] these may account for only a fraction of potential bypass signaling. Our findings here suggest that tissue engineering tools may provide unique opportunities to explore compensatory pathways mediated by the extracellular environment at the margins. Future work that employs patient-derived xenograft cells^[75] will likely yield a more physiologically relevant cell type and lead to outcomes more predictive of invasiveness and drug response for clinical applications. Further, while results here describe a discrete effect of matrix stiffness on GBM invasion, significantly future opportunities exist to explore a wider range of hydrogel stiffnesses, particularly below 8.8 kPa. Recent advances in hydrogel design also suggest the potential to explore not only stiffness but also the effect of matrix viscoelastic properties.^[76]

4. Conclusions

Given the short median survival time of glioblastoma patients, there is an acute need for in vitro platforms to facilitate rapid assessment of metrics of invasive behavior and therapeutic response. Approaches designed to address invasive spreading of GBM away from the tumor margins both before and after surgical debulking of the primary tumor offer the opportunity to consider the impact of biophysical properties of the tumor microenvironment on cell response. We report a bead-based hydrogel assay that provides the ability to trace long-term invasion metrics in a defined hydrogel environment in vitro. Using a series of GelMA hydrogels with or without matrix-bound HA, we report both biophysical (stiffness) and biomolecular (matrix vs soluble HA content) features both play important roles in GBM invasion within a fully 3D hydrogel network. Importantly, U251 GBM cells respond to their matrix environment, via changes in soluble HA production, to compensate for hydrogel environments lacking matrix-bound HA to stimulate their invasive potential. These findings also highlight the need for improved characterization of changes in matrix stiffness across the tumor mass and into the surrounding brain parenchyma as a means to improve hydrogel design targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Biophysical characterization of a family of GelMA hydro-gels. A) Elastic modulus determined via unconfined compression and B) diffusivity measured via FRAP as a function of total wt% (4, 5 wt%) and percent incorporated hyaluronic acid (w/w; 0%, 10%, 15%). ^: significant ($p < 0.05$) increase compared to 4 wt% hydrogel with identical fraction of HA. *: significant ($p < 0.05$) difference compared to hydrogels with same total wt% but different fractions of incorporated HA.

Figure 2.

A) U251 GBM cell-coated dextran beads within the GelMA hydrogel (Green: Actin cytoskeleton; Blue: nuclei). Scale bar: 50 μ m. B) Time-lapse (days 3, 5, 7) bright field images of U251 GBM cells spreading into the surrounding hydrogel matrix. Scale bar: 100 μ m. Metabolic activity of GBM cells within the series of C) 4 wt% (0%, 10%, 15% w/w HA) and D) 5 wt% (0%, 10%, 15% w/w HA) hydrogels. Averaged invasion distance of GBM cells within the hydrogel variants at E) day 3 and F) day 7. Invasion distance is significantly increased in lower elastic modulus hydrogels (4 wt%) and the absence of HA in the matrix. ^: significant ($p < 0.05$) increase compared to 5 wt% hydrogel with identical fraction of HA. *: significant ($p < 0.05$) difference compared to hydrogels with same total wt % but different fractions of incorporated HA.

Figure 3.

Influence of CD44-HA interaction inhibition. Reduced invasion was observed for GBM cells at A) day 3 and B) day 7 with CD44-HA interaction inhibition. However, limited differences were observed in metabolic activity with time in both the series of C) 4 wt% and D) 5 wt% hydrogels. #: significant ($p < 0.05$) decrease in invasion or metabolic activity with CD44-HA inhibition.

Figure 4.

Soluble HA concentration in culture media (normalized per cell) through day 7 as a function of total wt% (4, 5 wt%) and percent incorporated hyaluronic acid (w/w; 0%, 10%, 15%).

Soluble HA was significantly increased in hydrogels lacking matrix-bound HA. ^: significant ($p < 0.01$) increase compared to 5 wt% hydrogel with identical fraction of HA. *: significant ($p < 0.05$) difference compared to hydrogels with same total wt% but different fractions of incorporated HA.

Figure 5.

A,B) Gene expression profile of hyaluronic synthase (HAS-1, HAS-2, and HAS-3) at day 3 and day 7 in 4 wt% GelMA hydrogels as a function of percent incorporated hyaluronic acid (w/w; 0%, 15%) and CD44–HA inhibition. C,D) Gene expression profile of hyaluronidase (Hyal-1 and Hyal-2) in 4 wt% GelMA hydrogels as a function of percent incorporated hyaluronic acid (w/w; 0%, 15%) and CD44–HA inhibition.

Figure 6.

A) Western blot results for HAS-3 and Hyal-1 at day 7 in 4 wt% GelMA hydrogels as a function of percent incorporated hyaluronic acid (w/w; 0%, 15%) and CD44-HA inhibition. B) Quantifying the bands showed increasing trends of HAS-3 and decreasing trends of Hyal-1 with CD44 inhibition in GelMA hydrogels with matrix-bound HA, but no significant differences were observed.

Table 1

Primers used for gene expression.

Gene	Primer sequence (5'-xxx-3')	Citation
Hyal-1	Forward: CAT ATT GAG AAC CTA ATG CAC TCT G Reverse: GGA ATG AAT GGT GTC TGC TGT GG	[77]
Hyal-2	Forward: TTG TGA GCT TCC GTG TTC AG Reverse: GTC TCC GTG CTT GTG GTG TA	[77]
HAS-1	Forward: GGT GGG GAC GTG CGG ATC Reverse: ATG CAG GAT ACA CAG TGG AAG TAG	[32]
HAS-2	Forward: GTG GAT TAT GTA CAG GTT TGT GA Reverse: TCC AAC CAT GGG ATC TTC TT	[32]
HAS-3	Forward: CTC TAC TCC CTC CTC TAT ATG TC Reverse: AAC TGC CAC CCA GAT GGA	[32]
GAPDH	Forward: CCT TCC ACG ATA CCA AAG TTG Reverse: CCA TGA GAA GTA TGA CAA CAG CC	[78]