The Role of Hyaluronic Acid and Its Receptors in the Growth and Invasion of Brain Tumors

26

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Contents

Abstract

Malignant gliomas induce a complex cascade of changes in the extracellular matrix of the brain during their growth and invasion. This chapter highlights those changes involving hyaluronic acid, a glycosaminoglycan that constitutes much of the brain extracellular matrix, and the biophysical and biochemical effects those changes have on glioma cells. Signaling effects of hyaluronic acid receptors will be discussed, with a focus on CD44. The implications of CD44 enrichment in cancer stem cells will be discussed. Finally, because these interactions are highly dependent on the cellular microenvironment, we will review various in vitro cell culture platforms that have been used to model glioma cell motility and invasion.

Introduction

Malignant gliomas, particularly those classified as grade III or grade IV by the World Health Organization, are among the most fatal of all cancers. Grade IV glioma, also known as glioblastoma multiforme (GBM), is highly aggressive and recalcitrant to treatment, causing a dismal prognosis of 12–15 months after survival even with aggressive multimodal therapy (Siebzehnrubl et al. 2011). While the past two decades have seen much progress in understanding the origins and mechanisms of this aggressive cancer, these advances have not translated to a significant improvement in survival time. This is due in large

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part to the heterogeneity and genetic instability of glioma cells, which promote evasion of anti-cancer therapies. Current first line treatments for GBM include surgical resection of the tumor, chemotherapies such as the DNA-alkylating agent temozolomide, and radiotherapy. New targeted molecular therapies against tumor angiogenesis, such as bevacizumab, a monoclonal antibody against vascular endothelial growth factor, have shown some success in reducing tumor burden but have at best modestly increased survival time (Pàez-Ribes et al. 2009). Thus, the field continues to seek out new strategies to combine with those that are already used to form the most effective anti-cancer treatment possible.

Cells receive a plethora of signals from their microenvironment, including a variety of soluble autocrine, paracrine, and endocrine factors, as well as solid-state signals such as receptors on adjacent cells (e.g. cadherins) and the extracellular matrix (ECM), which cells engage through integrins and other adhesive receptors. These signals are then integrated by the cell to regulate polarity, motility, proliferation, cell fate, and a variety of other phenotypic characteristics. In cancer, both these microenvironmental biophysical signals and the way cells sense and respond to these signals are profoundly dysfunctional. Even in macroscopically static tissues cells exert forces against one another and the ECM, and the significant influence of this mechanical "context" on the resulting signal transduction has gained appreciation. These interactions are important during tumor invasion as well, as the microstructural arrangement of both ligands and steric barriers to migration greatly impact tumor cell motility. These ECM-based cues can in turn be remodeled by resident cancer cells, further perturbing native tissue homeostasis (Kumar and Weaver 2009).

These principles apply to the growth of GBM tumors, which is in large part dependent on glioma cell invasion through brain ECM. In vitro, the spreading area, motility, and proliferation of U373-MG and U87-MG cells is dependent on matrix stiffness (Ulrich et al. 2009). Neurosurgeons frequently use the high stiffness of brain tumors relative to normal brain tissue to identify appropriate resection planes, and these stiffness differentials have also been mapped by ultrasound. Finally, topologic structures in brain are thought to guide the migration of glioma cells; white matter fiber tracts and blood vessel basement membrane can act as tissue "highways" on which glioma cells invade rapidly to remote parts of the brain. Thus, an essential component to understanding the molecular mechanisms and potential points of intervention in GBM will be to clarify the biophysical inputs that cancer cells receive from their external environment, and how they interpret these inputs. Notably, brain ECM differs significantly from many connective tissues; whereas fibronectin, collagen, and vitronectin are essential elements of connective tissues and brain vasculature, brain parenchymal tissue is distinctly poor in protein, especially fibrillar proteins, and rich in glycosaminoglycans (GAGs). This chapter will focus on one of the key components of brain ECM, the GAG hyaluronic acid (HA). In GBM, HA is upregulated in the brain matrix, and HA receptors are overexpressed. We will now examine more closely how HA and its downstream signaling effects are relevant to the promotion of tumor growth and spread.

Hyaluronic Acid and Its Receptors

Matrix Properties of Hyaluronic Acid

Hyaluronic acid (HA), a linear GAG composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine, is essential to morphogenesis, tissue homeostasis, and wound repair throughout the body. Physically, due to its high density of anionic charge, HA is very hygroscopic and promotes tissue hydration and swelling. In brain, HA is a fundamental component of the ECM, as it serves as the high molecular weight template onto which many other hyaluronic acid binding proteins anchor (Fig. 26.1a). This class of proteins (variably called hyaladherins, HA binding proteins, and link proteins) shares a highly conserved HA-binding tandem repeat domain. The most prominent of these in brain is the lectican family of chondroitin sulfate proteoglycans, including versican, aggrecan, and neurocan.

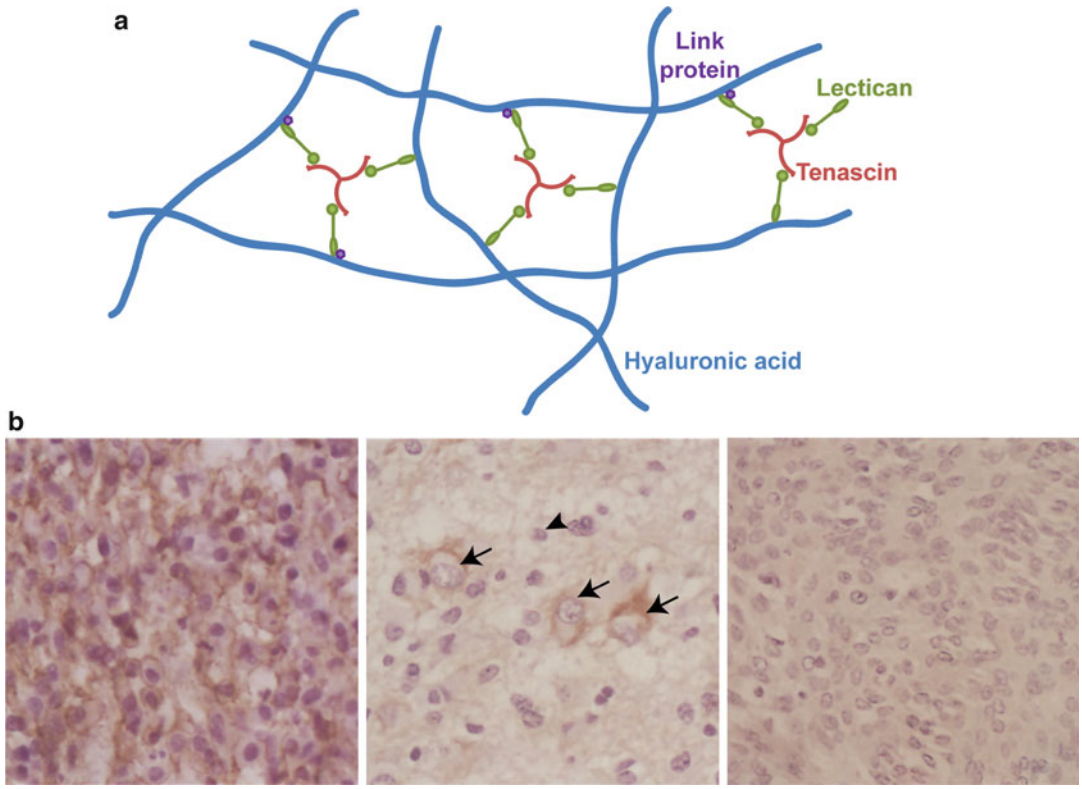

Fig. 26.1 Hyaluronic acid and CD44 are essential components of brain matrix and glioma invasion. (**a**) Schematic of HA architecture in brain. Brain matrix structure is based on high molecular weight hyaluronic acid (HA), which forms a hydrated network that is crosslinked by other biomolecules. Lecticans such as aggrecan, neurocan, versican, and brevican, are chondroitin sulfate proteoglycans that bind to HA at the N-terminus. HA-lectican binding is stabilized by link proteins. At the C-terminus, lecticans bind to the arms of tenascin-R, -C, and -W, which exist as trimeric or hexameric structures. (**b**) CD44 expression in the tumor microenvironment. Alterations in CD44 expression with tumor grade, demonstrated by immunohistochemical analysis of CD44 expression in human brain tumors by immunoperoxidase, with nuclei counterstained by eosin. An aggressive GBM tumor (*left panel*) displays intense staining of CD44. At the leading edge of the tumor (*middle panel*), neoplastic astrocytes with enlarged nuclei (*arrows*) display intense CD44 expression, while non-neoplastic cells (*arrowheads*) display little CD44 expression. In contrast, a noninvasive meningioma (*right panel*) shows weak CD44 staining. Magnification 400× (Ariza et al. 1995)

Chondroitin sulfate, the other glycosaminoglycan abundant in brain, is present as relatively minute sidearms on these proteoglycans. In contrast, HA is present as much longer chains, with molecular weight range of 100–1,000 kDa. Lecticans in turn bind to the third major class of brain matrix components, the tenascins, which are the main integrin-binding molecules in the brain. The biochemical makeup of brain stands in stark contrast to the collagen-based structures found in most tissues, so it is critical to recognize how these differences may affect cell adhesion and motility.

The exceptionally high molecular weight of HA is made possible by its unique mechanism of synthesis, which is orchestrated by the three HA synthases (HAS). Other GAGs are typically synthesized in the Golgi apparatus into short sidearms of proteoglycans, whose molecular weights are limited by vesicular transport. HA synthases, in contrast, are channel-like transmembrane proteins that sequester monosaccharides from the cytoplasm, add them onto the HA chain via glycotransferase domains, and extrude the growing linear chain out of the cell as it is synthesized. Thus, restrictions on the size of the HA

molecule are not imposed by biosynthesis and transport, and a single molecule can span several microns in length.

In the developing mouse cerebellum, HA organizes into fine web-like structures, which are hypothesized to aid in the migration of interneuron precursors and oligodendroglial cell types (Baier et al. 2007). Analysis of rat brain composition as a function of age shows that HA concentration peaks shortly after birth and then drops off in adulthood. However, in brain tumors, HA secretion is again elevated (Delpech et al. 1993), suggesting that glioma cells may hijack the natural HA-based motility mechanisms employed during development. While this hypothesis has not been clearly demonstrated in glioma cells, HAS2 overexpression in fibrosarcoma cells has been shown to have a direct link to tumorigenicity; cells transfected with *HAS2* demonstrated increased proliferation in a soft agar assay, and produced larger tumors in a nude mouse model (Kosaki et al. 1999). Whether HA elevation in glioma is due to HAS overexpression *per se* or some expression-independent enhancement in HAS activity remains unclear.

In the early stages of neural crest and brain development, levels of hyaluronidases (encoded by six *hyal* genes), the primary enzymes that degrade HA, are also at their peak as matrix turnover is necessary for cell migration. Increased HAS expression is only beneficial for cell migration if hyaluronidase is concurrently secreted; increased HA adhesive contacts must also be released for productive cell movement to occur (Enegd et al. 2002). Animal studies reveal that Hyal-2 overexpression facilitates tumor angiogenesis and formation in the HA-rich brain, but not in an HA-poor subcutaneous environment (Novak et al. 1999). Upon degradation of high molecular weight HA chains by hyaluronidase secretion from tumor cells, low molecular weight HA by-products of roughly 20 or fewer disaccharides are released, which then stimulate endothelial cells in neighboring blood vessels to undergo angiogenesis (Liu et al. 1996). While this mechanism is also necessary for the initiation of wound healing and matrix remodeling, it is one of the many HA-based signals that gliomas co-opt for aberrant growth and invasion. HA-based signaling mechanisms within glioma cells themselves are discussed in the next section.

Adhesion and Signaling Effects of Hyaluronic Acid Receptors

Chief among the diverse family of HA-binding adhesion proteins is CD44, a single pass transmembrane receptor that is upregulated in a variety of solid tumors, including brain tumors, and whose expression correlates with high glioma grade and poor prognosis (Ranuncolo et al. 2002). While the field has not converged on a single, dominant "canonical" CD44 pathway, the effects of HA binding with CD44 converge on proliferation, cell survival, and anti-apoptotic fate decisions through a variety of pathways (summarized in Fig. 26.2). CD44 does not have any intrinsic kinase activity, but rather executes its signaling effects by binding to kinases and other signaling molecules via its cytoplasmic tail, thereby recruiting these molecules to the cell membrane. While some CD44-HA signaling effects can be initiated by a single binding event, the high molecular weight characteristic of HA also serves to organize an activity-rich signaling center by bringing together many receptors and their downstream signaling partners. Indeed, CD44 tends to aggregate in protein-rich caveolae or lipid rafts.

At the gene level, *CD44* transcription is suppressed by binding of p53 to a non-canonical binding sequence in the *CD44* promoter (Godar et al. 2008). Godar et al. showed that many of the oncogenic effects of p53 loss are mediated by an increase of CD44-based survival signaling. Once CD44 is translated and brought to the cell membrane, CD44 complexes with a variety of pro-tumorigenic receptor tyrosine kinases to promote their kinase activity, including EGFR and ErbB2, TGFβ receptor, and c-Met/HGFR (Jung et al. 2011).

CD44 is capable of indirectly engaging the actin cytoskeleton through its cytoplasmic tail via the adaptor proteins ankyrin, and the ERM (ezrin-ridixin-moesin) family proteins. This interaction is essential for cell motility stimulated by phorbol

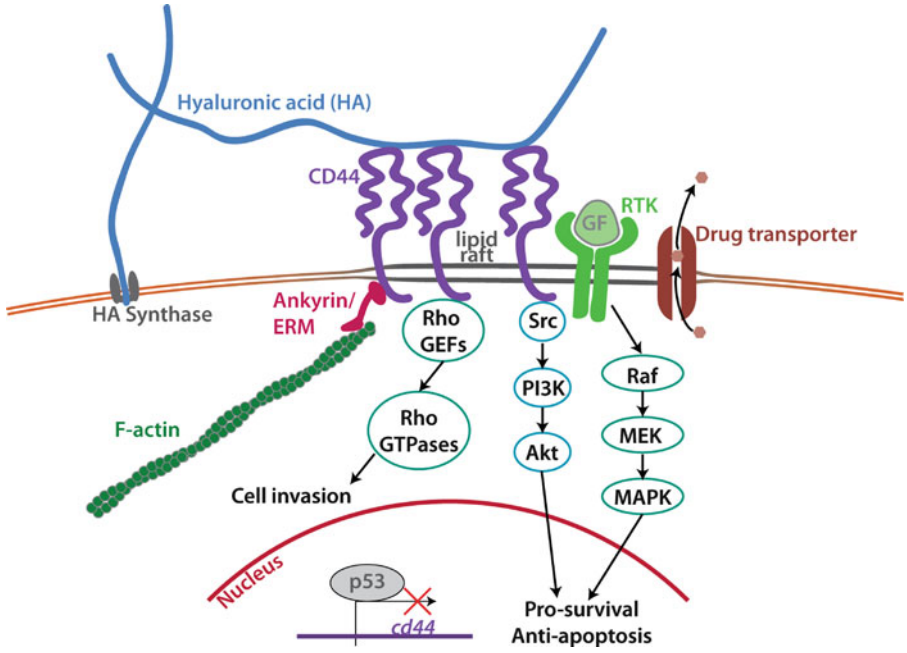

Fig. 26.2 Signal transduction mediated by hyaluronic acid (HA)-CD44 binding. HA is synthesized at the cell membrane by HA synthase, and extruded into the extracellular matrix. HA then binds to CD44, which can in turn associate with filamentous actin via ankyrin or the ezrin-ridixin-moesin (ERM) family proteins. RhoGTPases can also bind to the tail of CD44 via adaptor proteins. CD44 can associate in lipid rafts with receptor tyrosine kinases (RTKs) or multidrug efflux transporters on the cell membrane to promote their pro-survival and anti-apoptosis activity. All of these effects act in concert to promote cancer cell proliferation, motility, and chemoresistance. Transcription of *CD44* is kept in check by the tumor suppressor protein p53

ester, an analogue of diacyl glyceride (DAG). Legg et al. (2002) showed that the resulting activation of protein kinase C (PKC) triggers dephosphorylation of CD44 on Ser325 and phosphorylation on Ser291, then used fluorescence resonance energy transfer (FRET) to show that this dual change in phosphorylation reduces interaction between CD44 and ezrin. This dynamic control of ezrin association and dissociation via modulation of phosphorylation state is necessary for rapid chemotactic response to DAG gradients. Aside from anchoring to filamentous actin, CD44 also promotes cell motility by activating RhoGTPases. This occurs via recruitment of guanine nucleotide exchange factors (GEFs) to the cell membrane, facilitating interaction with their effectors, the Rho family of GTPases. Addition of soluble HA to cell culture medium quickly activates Rac-1 by recruiting the GEFs Tiam1 and Vav2, and induces lamellipodia formation within several minutes (Oliferenko et al. 2000) in both primary astrocytes and mammary epithelial cells. Similarly, in mammary epithelial cells, CD44-HA binding activates RhoA by recruiting p115 RhoGEF and myosin-mediated cell motility (Bourguignon 2008).

Other molecular mechanisms suppress the tumor-promoting effects of CD44 association with F-actin and activation of Rho GTPases. The most notable of these CD44-antagonizing molecules is merlin, the tumor suppressor protein encoded by the *NF2* gene, which blocks association with actin when bound to CD44. This also decreases CD44 affinity for HA, and thus reduces the intensity of downstream pro-tumorigenic signaling (Bai et al. 2007). In this way, CD44 acts as a molecular switch that promotes proliferation when ERM proteins are bound and quiescence when merlin is bound. Significantly, merlin is an essential antagonist of the Hippo signaling pathway, which attenuates apoptotic responses to oxidative stress and cytotoxic drugs. CD44 knockdown in

glioma cells reduces merlin phosphorylation (which is required for CD44 binding) and blocks the cell survival signals downstream of Hippo, thereby inducing p53 expression and increasing survival in an animal model (Xu et al. 2010).

In addition to interacting with actin binding proteins and RhoGTPases to promote cell motility, the cytoplasmic tail of CD44 also facilitates the formation of signaling complexes that ultimately promote cell survival. Namely, CD44 recruits several Src family non-receptor kinases such as Lyn (Lin 2001), Lck, Fyn, and c-Src (Bourguignon et al. 2001), which activate PI3K-Akt signaling. Integrins are perhaps the best known and widely studied cell adhesion receptors, so it is worth noting that the cytoplasmic tail of CD44 also interacts with integrin-based focal adhesion proteins. For instance, CD44 has been shown to complex with focal adhesion kinase (FAK) in a lung cancer cell line, and cells stimulated by HA express increased activated (FAK), which in turn augments both the PI3K and MAPK pathways (Fujita et al. 2002). Thus, this extensive set of interactions allows CD44 to exert its signaling effects even in the absence of any intrinsic kinase activity.

Finally, RHAMM (receptor for hyaluronic acid mediated motility) is another HA receptor that has been studied for its pro-tumorigenic effects. The functions of RHAMM and CD44 appear to be inextricably linked; many roles of RHAMM involve supporting CD44-HA binding and downstream signaling. Additionally, in an arthritic mouse model, increased RHAMM expression has been shown to phenotypically complement CD44 gene suppression, as CD44 knockout mice develop normally (Nedvetzki et al. 2004). While the two receptors share many of the same binding partners, RHAMM does have other tumorigenic roles independent of CD44; for example, its other primary role is to facilitate the assembly of mitotic spindles required for cell division by assisting in microtubule nucleation. This role, independent of HA binding functions, is known to be especially active in breast cancer cells, where it is proposed that overexpressed RHAMM drives aberrations in mitotic spindles and thus supports genomic instability.

Significance of CD44 in Cancer Stem-Like Cells

Historically, the progression of GBM has been framed in terms of clonal evolution models, in which cellular heterogeneity arises from the stochastic accumulation of different mutations by different cells. More recently, a new prevailing paradigm is emerging in which GBM is thought to progress according to a cancer stem cell hypothesis, in which a specialized and perhaps rare subpopulation possessing the hallmark stem cell characteristics of self-renewal and multipotency give rise to a heterogeneous bulk tumor population. While components of this model remain somewhat controversial, a preponderance of evidence now supports that a stem-like subpopulation of cells exists within the heterogeneous tumor population that has much higher tumorigenic potential than the other cells. For example, only specific cell subpopulations of primary tumors are capable of histologically recapitulating human tumors when orthotopically implanted at low numbers in immunocompromised mice. These subpopulations often share key features of normal neural stem cells such as expression of neural stem cell markers (e.g. nestin), the ability to form clonal neurospheres, strong preference for laminin-based ECMs, and, most critically, the ability to self-renew and differentiate into cells positive for neural, astrocytic, and oligodendrocytic markers. As is commonly done in the field, we will refer to this stem-like population as brain cancer stem cells (BCSCs).

BCSCs can be selected from a primary tumor by culturing tumor explants in neurobasal medium supplemented with growth factors, which is also used as a neural stem cell growth medium. Some fraction of the tumor cells will form clonal neurospheres under these conditions, and these clones satisfy specific criteria including expression of specific markers, multipotency, and – critically – the ability to histologically recapitulate GBM when orthotopically implanted into immunocompromised mice. These cells are designated as BCSCs, and significantly, this population is enriched in CD44 expression. One possible functional consequence of CD44 overexpression is the promotion

of multidrug resistance proteins on the cell membrane, which confer chemoresistance by pumping cytotoxic agents out of the cancer cell (Toole and Slomiany 2008). An alternative method of isolating cancer stem cell populations involves collection of "side population" cells, which are defined by low uptake of Hoechst dyes due to high expression of ABCG2 (BCRP) and ABCB1 (MDR1) drug transporters of the ABC (ATP-binding cassette) family (Hirschmann-Jax et al. 2004). This expression pattern partially accounts for the highly chemoresistant characteristics of BCSCs compared to their bulk tumor cell counterparts. BCSCs also divide, or cycle, less rapidly than bulk tumor cells, reducing the uptake and efficacy of anti-cancer drugs whose mechanism of action requires cell division.

Flow cytometry and histological analysis of human brain tumors reveals that glioma cells express a variety of these drug transporters, which are expressed more frequently in high grade tumors (Calatozzolo et al. 2005). In a malignant peripheral nerve sheath tumor cell line, CD44 forms a stable complex with ABCG2 in the plasma membrane (Slomiany et al. 2009). This complex is disrupted by adding oligomeric HA, which competes with endogenous high molecular-weight HA for CD44 binding, and leads to internalization of both CD44 and ABCG2. Finally, cells treated in this way are rendered more susceptible to apoptosis induced by the anti-cancer DNA intercalating agent doxorubicin. Since ABCG2 is highly expressed in BCSCs (Bleau et al. 2009), strategies to disrupt the association of CD44 and multidrug transporters expressed on BCSCs may prove to be effective ways of improving the effectiveness of chemotherapy treatments for malignant gliomas. The role of HA in the tumor matrix on promoting multidrug resistance in BCSCs has not yet been investigated.

While CD44 is increasingly accepted as a BCSC marker, the functional significance of CD44 enrichment in BCSCs remains unclear. Recently, Jijiwa et al. (2011) began to investigate these questions through the identification and characterization of CD44 variants in BCSCs. While CD44s is the standard isoform of the receptor, variant isoforms can be formed by alternative splicing of exons encoding the middle stalk region of the receptor; all isoforms contain the same intracellular domain and HA-binding domain. Expression of the variant isoform CD44v6 has long been known to be exclusively expressed by cancer cells of other tissues where it promotes cancer malignancy and invasiveness, but now it is known that CD44v6 is also expressed by BCSCs (Jijiwa et al. 2011). CD44v6 promotes cell survival signals by Akt phosphorylation when it binds to its secondary ligand, osteopontin. Thus, the further exploration of the functional role of CD44 expression in the BCSC specialized cell population will surely unlock greater understanding of the mechanisms of GBM progression.

In Vitro Culture Models of Brain Tumor Invasion

Any cell-level in vitro study of GBM must strike a balance between interpretability and physiological mimicry. On one extreme, cell culture paradigms employing two-dimensional tissue culture polystyrene (TCPS) surfaces are convenient and widely used, but they do not recapitulate the myriad microstructural, biophysical, and biochemical features of the in vivo brain microenvironment. When one's goal is to systematically test the regulatory role of specific, defined features of this microenvironment, it may be advantageous to use these highly defined albeit reductionist platforms. In other cases, it may be desirable to incorporate as many brain matrix characteristics as possible. We now briefly review strategies that have been used to study GBM in a cell-scale in vitro setting, with a focus on the incorporation of HA in these systems. In addition to the models described here, there are also a variety of in vivo animal models that are available for studying heterotypic cell interactions (e.g. with endothelial cells) or more clinically relevant endpoints (e.g. tumor size or animal survival).

2D Culture Models

The simplest approach for studying the effects of soluble factors such as HA is to place them in the

cell culture medium itself. This format has been vitally important in identifying the many biochemical signaling effects of HA. However, from a biophysical standpoint, it does not mimic important structural aspects of large matrix molecules such as full-length adhesive proteins or full-length HA. To study the adhesive effects of these matrix macromolecules, they can be adsorbed onto glass or TCPS surfaces. However, the disadvantages to this approach are that the stiffness of these surfaces is orders of magnitude higher than most tissues including brain, that adsorption can alter the biological activity of the adsorbed ligand, and that the adsorption is nonspecific. To mitigate many of these disadvantages, polyacrylamide and other polymer hydrogel substrates have been widely used to create matrix materials with finely tunable stiffness (Wang and Pelham 1998) by varying the ratio of the monomer and crosslinker (e.g. acrylamide and bis-acrylamide). Matrix proteins such as fibronectin or collagen can be covalently attached to the hydrogel surface, often with heterobifunctional coupling agents such as sulfo-SANPAH (sulfosuccinimidyl-6-(40-azido-20-nitrophenylamino) hexanoate) which may be conjugated to the gel surface with UV irradiation and to ECM proteins via NHS-ester chemistry. Aside from the selective covalent attachment of desired proteins using this chemistry, serum proteins do not adsorb onto polyacrylamide, allowing for a highly controlled biochemical and biomechanical surface.

More recently, soft lithographic techniques have emerged as a powerful method to selectively pattern precise 2D geometries of adhesive proteins set on an adhesion-resistant background (Chen et al. 1997). Typically, a molded stamp of polydimethylsiloxane (PDMS) is coated with adhesive protein, and stamped onto a bioinert layer. The effects of cell shape, cell size, and cell-cell interactions have been well explored with this technique, as well as "1D" studies of cell migration on thin confined paths.

Cell motility is another important characteristic of cancer cells that can be measured in vitro. Time-lapse microscopy can be used to track the paths of migrating cells over time, yielding metrics such as cell speed and persistence length. In trans-well (Boyden chamber) assays, which enable the assessment of invasive cell motility, cells are seeded on top of polymer membranes with pore diameters on the same length scale of the cell. Cells are then challenged with a chemoattractant placed in the bottom compartment, thus inducing migration through the pores. The number of cells that successfully traverse a pore and arrive in the bottom chamber in a given time may then be used as a metric of invasive potential. A wide range of pore sizes is available, and the filters can be coated with specific ECM proteins to assess the effect of these proteins on migration.

While 2D models may be considered reasonable representations of certain native tissue geometries such as epithelial and endothelial monolayers, many other cell types, including glioma cells, are fully ensconced by ECM and/or cells in all three dimensions. Moreover, multiple lines of experimental evidence have revealed that glioma cell behavior often depends strongly on matrix dimensionality, i.e. 2D vs. 3D (Beadle et al. 2008). One way to create a 3D environment directly from a 2D surface is to use "sandwich" cultures, in which cells are first cultured on a 2D surface, and then another matrix layer is placed on the apical side of the cells. This method is widely used for the culture of mammary epithelial cells between layers of Matrigel and has been shown to reproduce defining phenotypic features of 3D matrices (Lee et al. 2007). In addition to its experimental convenience, this geometry recapitulates key features of anatomical interfaces along which glioma cells invade, such as the basement membrane of blood vessels and the glia limitans externa.

3D Culture Models

Many tissue architectures, including that of the brain parenchyma, are more accurately recapitulated by a "true" 3D structure in which cells are embedded within, rather than sandwiched between, ECM. This has significant implications for a number of cell behaviors relevant to glioma invasion, particularly cell motility. In 2D, lamellipodium formation at the leading edge is not

geometrically constrained, and productive forward movement of the cell body is restricted by rupture of adhesions at the trailing edge. However in 3D, cells must either exert force to extrude through voids in the matrix, or enzymatically degrade the matrix to clear a path for productive movement. However, the use of 3D matrices presents unique challenges not encountered in 2D culture, including limited throughput, potentially uneven exposure to oxygen and soluble factors, reduced suitability for high-resolution imaging, and difficulty harvesting cells at high numbers for post-hoc analysis.

As described above, the task of imaging individual cells using light microscopy can be problematic in 3D cultures; images contain optical contributions from material above and below the focal plane, and cells may migrate into and out of focus. To overcome these and other challenges, multicellular tumor spheroid models are a convenient and widely-used method of tracking invasive behavior. Spheroids are formed by culturing cells in hanging droplets, in which the cells aggregate into dense spherical masses. The spheroids are then implanted during, or less commonly after, gel solidification. Local nutrient and oxygen gradients are automatically imposed by the geometry of the spheroids, and this induces cells on the periphery of the spheroid to invade outward if the matrix is permissive to migration (analogous to the familiar scratch wound assay in 2D). These built-in gradients are a major advantage of this method, which also mimics some aspects of multicellular tumor growth. Although single cells cannot be tracked for long periods of time, migration of the overall population can be monitored simply by tracking the invasive radius or projected area. In a variation on this paradigm, cells can also be cultured on the surfaces of microcarrier beads and then implanted, which has the benefit of standardizing aggregate size and limiting necrosis but may not recapitulate important radial cell-cell interactions found in tumors.

In terms of design of matrix materials, the most basic 3D matrices are biologically-derived protein gels such as collagen, fibrin, and Matrigel. While all of these model systems continue to serve as important discovery platforms for GBM, they have several limitations. First, matrix properties cannot be tuned over a wide range of stiffnesses, as these materials are intrinsically quite soft and cannot easily be stiffened or softened independent of other critical properties. For example, manipulation of matrix stiffness by increasing protein density also alters ligand density and the pore size of the matrix, which may sterically hinder migrating cells and alter solute diffusion. To overcome the first problem, our group has developed a collagen-agarose hybrid system, in which the addition of inert agarose stiffens the matrix without simultaneously adding more ligand density (Fig. 26.3b) (Ulrich et al. 2011). However, in this system, agarose also introduces steric barriers and restricts cell-directed remodeling of the collagen fibers.

Biologically-derived materials that do not form a gel on their own can (and in some cases must) also be covalently modified and crosslinked in order to form a solid hydrogel. For instance, HA does not spontaneously assemble into gels but can be made to do so if chemically modified and covalently crosslinked (Ananthanarayanan et al. 2011; Burdick and Prestwich 2011). By adding reactive sites on an otherwise unreactive biopolymer, stiffness and density can be controlled by extent of functionalization, crosslinking, and the molecular weight of the starting material (Fig. 26.3c). HA can also be used as a scaffold to embed matrix proteins (e.g. fibronectin, laminin) that do not easily assemble into gels on their own.

An alternative strategy to native ECM formulations is the use of synthetic polymers as a "blank slate" on which to build modular bioactive components. Both the polymerization and crosslinking chemistries of polyacrylamide, the most commonly used 2D synthetic hydrogel, are highly cytotoxic, rendering this material inappropriate for hydrogel assembly around cells. Instead, pre-polymerized materials such as polyethylene glycol (PEG) or polyvinyl alcohol are used in conjunction with more biocompatible crosslinking chemistries. Biocompatible photoinitiators and Michael Additions are broadly used for these crosslinking reactions. To add cell-adhesion functionality, full-length proteins can

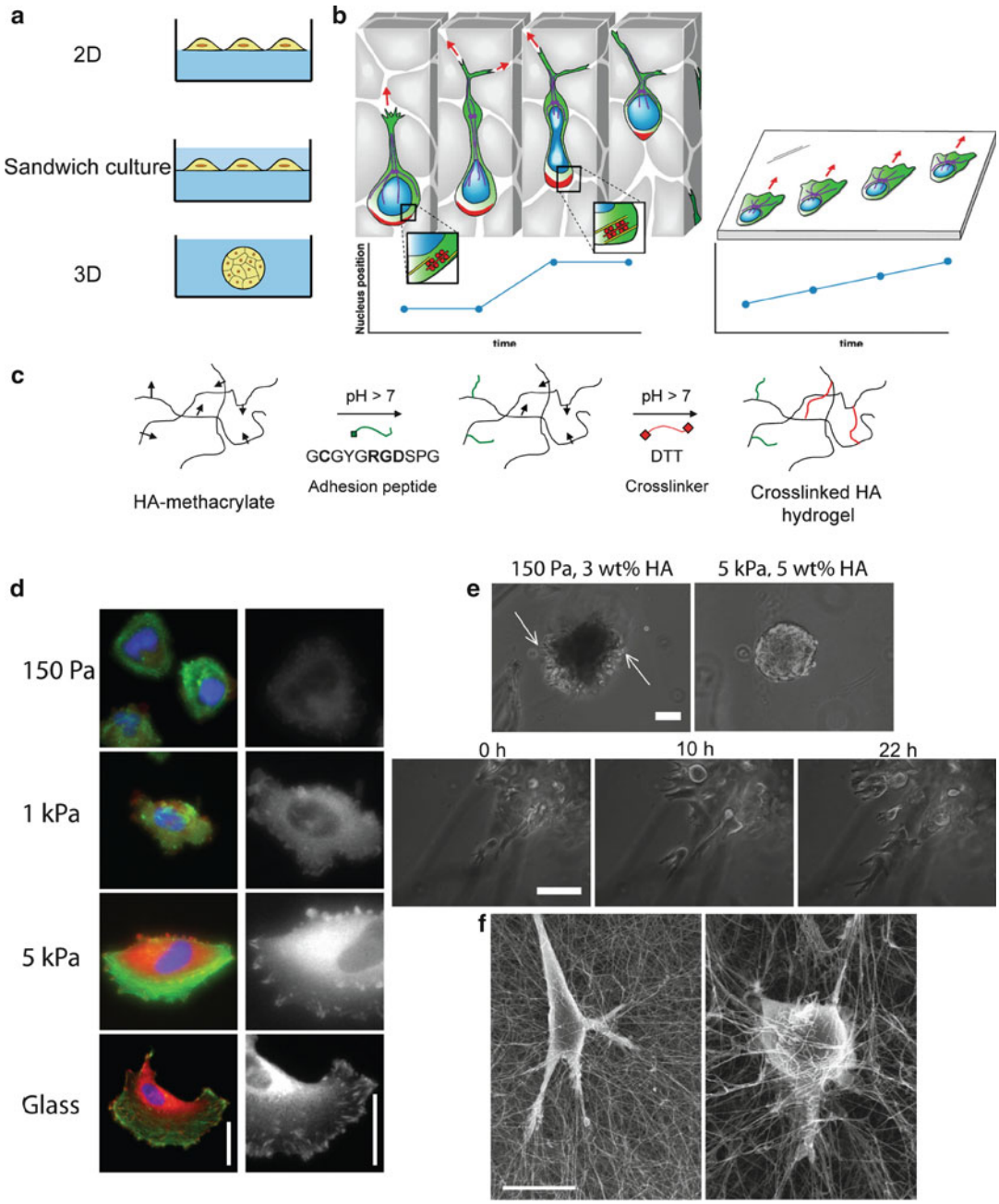

Fig. 26.3 In vitro cell culture models of GBM. (**a**) Schematic of various in vitro geometries. In 2D, cells lie on a flat monolayer, either on glass, tissue culture polystyrene, or an engineered or reconstituted matrix. In sandwich culture, they lie at an interface between two layers of matrix. In 3D, cells are surrounded on all sides by matrix either as single cells or in a spheroid as diagrammed. (**b**) Schematic of differing cell migration in 3D environment of brain (*left*) versus 2D (*right*). In 3D, glioma cells extend highly dynamic and branched leading protrustions to extrude cell bodies and nuclei through small voids between neighboring cells in order to migrate. In 2D, cell migration is not spatially hindered, and lamellipodial protrusion occurs freely, resulting in smooth gliding movement (Beadle et al. 2008). (**c**–**e**) Synthetically modified matrix based on hyaluronic acid (Ananthanarayanan

be embedded in the matrix prior to crosslinking, or the polymer can be covalently functionalized with bioadhesive peptide sequences. Finally, enzymatically degradable peptide sequences can also be incorporated into the crosslinks to allow for matrix degradation as cells proliferate and/or migrate.

More recently, some investigators have adapted a third, distinct approach based on the use of decellularized matrices (Fig. 26.3f), in which native tissue is denuded of cells using chemical detergents, which in the case of brain leaves behind matrix proteins, GAGs, and other brain-specific components (Crapo et al. 2012). This material can also be liquefied by pepsin digestion, but re-assembles after injection in vivo. This approach can have great advantages over building matrix scaffolds from the ground up, but faces unique challenges such as immune response, residual DNA, and sample-to-sample variability, though these are of greater concern for regenerative medicine applications than in vitro studies or in vivo implantation into immunocompromised animals. These materials have not been extensively explored in the context of glioma invasion, but may hold great promise as a sort of middle ground between reconstituted matrix preparations and in vivo paradigms. Given the history of reconstituted and synthetic ECM scaffolds, it is reasonable to expect that future efforts will focus in part on manipulation of properties such as porosity and matrix stiffness in these decellularized systems.

Discussion

CD44 was first discovered in the 1980s by the immunology community, which quickly found that the cell surface molecule is involved in a variety of diverse functions such as leukocyte homing and T-cell activation. By the early 1990s, cancer researchers began to find yet another CD44 function: that it is differentially expressed in many cancers, and plays an important role in cancer invasion and metastasis. Today, the quest to fully understand the processes of this complex molecule is still not complete; much remains to be discovered about not only the exact signaling pathways in which CD44 is involved, but to also understand how these numerous and at times conflicting signals are interpreted or exploited by cancer cells.

In GBM, aberrant CD44-HA mediated pathways play a critical role in aiding, and perhaps even driving, the highly motile, malignant, and chemoresistant properties of gliomas that make them so fatal. In this review, we have discussed how elevation of HA secretion in tumor matrix and high CD44 expression in glioma cells combine to promote cell invasion and survival in GBM. In the specialized BCSC population, we raised the prospect that the high expression level of CD44 may be more than simply a correlative marker and may play key functional roles in maintaining proliferation and chemoresistance patterns that are hallmarks of malignant gliomas.

Fig. 26.3 (continued) et al. 2011). (**c**) Chemical modification scheme, in which synthetically methacrylated HA can react with free thiol groups, such as those on cysteine-containing resides or the bifunctional crosslinker dithiothreitol (DTT) (Ananthanarayanan et al. 2011). (**d**) U373-MG cells on 2D HA functionalized with RGD show a stiffness-dependent response to spreading, actin (*green*) stress fiber formation, and vinculin-based focal adhesions (*red*). (**e**) Spheroids of U373-MG cells embedded in RGD-functionalized HA demonstrate dependence on stiffness and density of hydrogel (*top panel*). In the 150 Pa 3 wt% gel, cells migrate outward in a highly branched and dynamic manner (Ananthanarayanan et al. 2011). (**f**) Collagen-agarose hybrid matrices. The collagen-agarose system is another example of a biologically derived matrix. Scanning electron micrographs of pure collagen matrix (*left panel*) show that a U373-MG glioma cell can exert forces needed to bundle collagen fibrils and spread. In contrast, the addition of agarose prior to collagen gelation intercepts this bundling process, and the cell remains rounded (Ulrich et al. 2011)

Finally, we discussed the rational design of in vitro GBM studies, ranging from highly simplified models to complex ones that incorporate important structural, biochemical, and biomechanical aspects of native tissue.

This body of knowledge leaves many open questions unanswered. Among the most critical of these is whether CD44 expression is a requirement for GBM malignancy, or whether it is a downstream effector that happens to promote further cancer invasion. Thus far, several clues suggest that CD44 may contribute in significant ways to glioma progression, such as its previously discussed role in executing many of the cancer-promoting effects of *p53* loss. However, the extent to which CD44 alone is intrinsically responsible for triggering tumorigenesis is not yet clear. This question intersects with our emerging understanding of the tumor-initiating role of BCSCs, which often happen to overexpress CD44. While CD44 supports chemoresistance in these cells, it remains unknown whether CD44 overexpression in this subpopulation causally drives this process. To decipher these puzzles, combined approaches must be brought to bear from the fields of cell-ECM biology, bioengineering, and cancer biology. The list of questions these multidisciplinary approaches could help address is a long one. For example, what are the individual roles of CD44 isoform expression on GBM progression? How do CD44-matrix linkages and their downstream signaling crosstalk with other important pathways in brain, including aberrant growth factor and integrin signaling? To what extent does the CD44 receptor transduce force-based signals, as integrins have long been recognized to do? Finally, as animal models of GBM become increasingly sophisticated, researchers will have more powerful tools to study the impact of these questions in an in vivo system, and ultimately the genetic and molecular origins of GBM.

While animal models are critical to cancer research, in vitro studies are also a powerful platform to study cell-ECM interactions in a controlled and manipulatable manner. CD44 and other cell surface molecules act as the interface through which the cell communicates with its surroundings, so brain ECM composition and structure, and cancer-induced changes in these properties, compose an essential piece of the puzzle. Therefore, another challenge in this field is the incorporation of brain-specific features into ECM models used to study GBM growth and invasion, including those having to do with ligand type and presentation and scaffold mechanics. With our exploration of various in vitro platforms for studying glioma cell behavior, we reviewed some methods developed to isolate and study certain aspects of the distinct microstructural and biophysical properties of brain. A central limitation of the current generation of HA-based scaffolds is the lack of fine microstructural and topological control, with the creation of multicomponent, hybrid matrices with the same molecular arrangements as those found in the brain remaining a particular challenge. Finally, there remains a great need for methods to incorporate larger anatomic structures in brain, such as blood vessels and white matter fiber tracts. In this regard, decellularized matrices may offer an important and relatively unique opportunity to perform "top down" matrix engineering, in which post hoc synthetic modifications on the matrix could be made to study their effects on glioma cell motility in an environment that retains all of the native ECM of brain.

Ultimately, these future findings and methods can be leveraged to design new therapies that make anti-cancer regimens more effective. As we have discussed, several studies have begun to explore the HA-CD44 interaction as a potential "druggable target," with promising results. Examples include addition of oligomeric HA to competitively bind and reduce the receptor clustering effect of high molecular weight HA, or addition of hyaluronidase to enzymatically cleave it. As discussed earlier, these strategies should be systematically tested in increasingly physiomimetic models of human GBM, using not only the endpoints of cell proliferation and apoptosis which can be screened in vitro, but also heterotypic cell-cell interactions, angiogenesis, and mean survival time which can only be tested in vivo.

Perhaps an even greater challenge than understanding how cancer cells synthesize and respond

to signals through one receptor such as CD44 is to understand how they fit in with the greater picture, composed of an entire slew of signals from other adhesion and growth factor receptors, and biophysically-imposed constraints. In particular, the great focus of the field on the dominance of integrin-based adhesion and tumorigenic signaling calls into question how integrin-ligand and CD44-HA receptor-ligand pairs might interact, and what relative role they have in GBM progression. The field's attempts to answer these questions have the potential to not only inform greater therapeutics to treat GBM, but also to understand and treat other cancers throughout the body.

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