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Kundu S, Forsberg-Nilsson K

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Glycosaminoglycans and Glioma Invasion

Soumi Kundu, Karin Forsberg-Nilsson

Abstract: There is a great need for novel therapies to target malignant glioma, a disease with an often dismal prognosis. One of the hallmarks of malignant glioma is its efficient invasion of the healthy brain parenchyma, which leads to rapidly recurrent disease upon surgical removal of the original tumour. To be able to establish new tumours at a distance from the original neoplasm, glioma cells must detach, migrate through the microenvironment, settle, and proliferate in their new location. This includes changing adhesive characteristics, breaking down extracellular matrix molecules (ECM), and perturbed growth factor signalling. Investigations of the glioma-specific ECM composition may therefore provide new insights into glioma infiltration. In this review, we focus on glycosaminoglycans, important components of the ECM that are long unbranched polysaccharides composed of repeating disaccharide units. We discuss the roles for hyaluronan, one of the major brain ECM molecules, and that of the proteoglycans, heparan sulphate proteoglycans (HSPG) and chondroitin sulphate proteoglycans (CSPG), in glioma biology. Heparan sulphate (HS) and chondroitin sulphate (CS) chains act together with a wide variety of bioactive molecules, and these interactions depend on the HS and CS sulphation patterns. HS and CS chain modifications are implicated not only in normal development and homoeostasis but they also play important roles in pathological conditions including cancer. Dysregulated glycosaminoglycans, their biosynthetic and degradation enzymes as well as the proteoglycan core proteins are known to affect several stages of tumour progression, angiogenesis, and metastasis. Finding the specific characteristics of tumour cells that confer this infiltrative capacity of glioma may offer new avenues for drug development. **Eur Assoc NeuroOncol Mag 2014; 4 (2): 75–80.**

Key words: heparan sulphate, chondroitin sulphate, extracellular matrix, tumour invasion

Glioblastoma

Gliomas are classified according to WHO criteria in grades I–IV where glioblastoma is the most aggressive and also the most common malignant primary brain tumour among adults. Every year, approximately 3 per 100,000 individuals are diagnosed with glioblastoma [1] for which the standard treatment is surgery followed by radiation and chemotherapy. Even though the introduction of temozolomide has somewhat prolonged life expectancy, the outcome is still poor with a median survival of only ~15 months [2].

In addition to the classical WHO classification, glioblastoma can be further divided into subtypes, using a molecular-based taxonomy. This aims at better describing the heterogeneous nature of these tumours. The term glioblastoma multiforme (GBM) is used synonymously with glioblastoma to emphasize the intra-tumour and inter-tumour diversities. By means of a combination of mutational and expression profiling data several molecular subgroups have been described [3–5]. From the Cancer Genome Atlas (TCGA) data collection [5], GBM was divided into the following subtypes: (1) proneural, (2) neural, (3) classical, and (4) mesenchymal. An even more recent classification by Sturm et al [6] added the methylation patterns in the subtype analysis, and also included paediatric GBM. In addition, a description of the landscape of somatic mutations in GBM [7] provides further information on subtypes, survival, and increased understanding of the disease at the molecular level. Taken together, a solid base for patient stratification is becoming possible due to the increased availability of mutational analysis and expression data for GBM.

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From the Science for Life Laboratory, Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Sweden

Correspondence to: Karin Forsberg Nilsson, PhD, Department of Immunology, Genetics and Pathology, Uppsala University, Rudbecklaboratoriet, 75185 Uppsala, Sweden; e-mail: karin.nilsson@igp.uu.se

Glioblastoma Invasion Is a Major Clinical Problem

A typical feature of GBM is the extensive infiltration of tumour cells into the healthy parenchyma that makes complete resection impossible. This feature is common for all diffuse astrocytic tumours, but it is a particular problem in GBM [8]. After surgical removal, tumour recurrence within a distance of several centimetres from the original tumour site often occurs [9]. The high motility of glioma cells is underscored by their ability to spread even to the contralateral hemisphere via the corpus callosum. However, in contrast to its efficient invasion within the brain, GBM rarely metastasizes via the cerebrospinal fluid [10].

Therapies to target GBM invasion are highly warranted because of its efficient migration, eg, along white-matter tracts [11]. For a tumour to spread away from the original neoplasm, cancer cells have to detach and migrate through the parenchyma, which includes breakdown of several extracellular matrix molecules. It is well-known that glioma cells express cell adhesion molecules that facilitate the invasion process [12]. Tumour cell migration needs to be accompanied by matrix degradation, which is carried out by a variety of enzymatic processes [13]. This leads to ECM remodelling since the migrating tumour cells lay down *de novo* ECM of their own, composed primarily of ECM molecules supporting migration [14].

As of today, novel therapeutic approaches mainly target the cancer cells *per se* and focus less on the surrounding, non-tumour environment. In fact, most clinical trials have been directed against the bulk of cancer cells and few drugs have been tested that target the invasive mechanisms. Attempts at reducing matrix metalloproteinase (MMP) levels in glioma with marimastat were not successful since the combination of marimastat and temozolomide was approximately equivalent to temozolomide alone and also caused toxic effects [15, 16]. Another example are clinical trials with cilengitide, an inhibitor of integrin $\alpha v\beta 3$ and integrin $\alpha v\beta 5$ [17] that may affect both growth and spread of tumour cells as well as angiogenesis.

However, in February 2013, it was announced that the phase-III clinical trial of cilengitide did not increase overall survival over standard treatment comprising radiation and temozolomide. In light of these failures, more targets specific for tumour cell invasion are needed, based on mechanisms responsible for tumour cell infiltration. In this review, we focus on the role of glycosaminoglycans because they are major components of the extracellular matrix and important players in the invasive processes.

The Extracellular Matrix of the Healthy and Malignant Brain

Several aspects of ECM biology are different between the brain and non-CNS tissues. Collagen-containing basement membranes that are typical for the extra-CNS tissue are only present in the meninges and around blood vessels in the normal brain. Basal lamina extensions from the vessels in the subventricular zone form structures called fractones [18] that have been suggested to enrich growth factors and other HS-binding signalling molecules in the neurovascular niche, where adult neural stem cells reside. Cancer stem cells (CSC) in glioblastoma share many properties with normal neural stem cells, such as their ability for self-renewal, and they are highly motile [19]. The perivascular niche in glioma is believed to harbour CSCs that are supported by trophic factors from the vasculature [20]. Sequestration of growth factors by HSPG in the tumourigenic niche could thus maintain cancer stem cells in a tumourigenic niche with similarities to the normal neurogenic niche (reviewed in [21]). In contrast to the normal brain glioblastoma has greatly increased levels of collagen [22] and the importance of the collagen structure for glioblastoma neovascularization and tumour growth has recently been reported [23].

In the adult brain, the extracellular matrix regulates structural and functional plasticity, partly through the brain-specific dense ECM structures called perineuronal nets. These were first described by Golgi (reviewed in [24]) and are composed mainly of chondroitin sulphate proteoglycans, hyaluronan, Tenascin-R, and Sema3A [26]. The perineuronal nets restrict reorganization of process formation mainly through inhibitory CSPG and Sema3A. CSPGs are highly up-regulated in glioma [27] but their role in glioma invasiveness is not fully elucidated [28, 29], as will be discussed below.

Glycosaminoglycans Are Major Components of the Brain ECM

Glycosaminoglycan (GAG) is the common term for linear polysaccharides that are composed of repetitively appearing disaccharides consisting of an N-acetylated or N-sulphated hexosamine together with either uronic acid (glucuronic acid or iduronic acid) or galactose. These molecules are highly conserved during evolution, which points at their ubiquitous functions in many biological processes [30]. Hyaluronan, a major constituent of the extracellular matrix (ECM), is a large, unbranched glycosaminoglycan that lacks sulphate groups. The glycosaminoglycans heparan sulphate (HS), chondroitin sulphate (CS), dermatan sulphate (DS), and keratan sulphate (KS) make up the proteoglycans, consisting of core proteins covalently linked to one or more glycosaminoglycan chains. The glucosaminoglycan components of proteoglycans are sulphated in various positions, with a differing disaccharide composition. Dermatan sulphates (DS) have iduronic acid, while chondroitin sulphates (CS) lack these residues. Keratan sulphates (KS) have no uronic acid and are instead made up by N-acetylglucosamine and galactose units. Proteoglycans are either attached to the plasma membrane, found in vesicles inside cells, or exported to the extracellular space [31]. Because of the high abundance of hyaluronan, heparan sulphate, and chondroitin sulphate in the brain, relative to dermatan sulphate and keratan sulphates, this review will focus on the first 3 types of molecules.

Hyaluronan

Hyaluronan (HA) is the principal component of the brain ECM with a unique composition, and it does not undergo sulphation and epimerization [32, 33]. HA content is elevated in primary brain tumours [34]. In contrast to other GAGs, HA is not synthesized in the Golgi apparatus. Hyaluronan synthase is the primary biosynthetic enzyme in mammals, which occurs in 3 isoforms synthesizing HA polymers of different lengths. The enzyme is located in the plasma membrane and catalyzes polymerization as well as translocation of HA out of the cell [35, 36]. In most tissues, HA is rapidly degraded by hyaluronidase (Hyal). Mammalian Hyal has overlapping substrate specificity and is known to degrade HA and CS and, to some extent, DS. Hyaluronidase acts in concert with 2 other exoglycosidases (removing sugars from the non-reducing end [37]). Human glioblastoma expressed levels of hyaluronan synthases above that of normal cells [38, 39] and over-expression of hyaluronan synthase-2 reduced growth of murine glioma, but only if hyaluronidase was concomitantly present [38]. Furthermore, hyaluronan synthase has been associated with increased receptor tyrosine kinase activity [40] and hyaluronan synthase-2 mRNA levels were higher in GBM than in normal brain [41]. This implies an important balance between synthesis and breakdown of HA in brain tumour biology.

HA can reach high molecular mass, consisting of 25,000–30,000 disaccharide repeats under normal conditions, and it attains complex secondary and tertiary structures regulating physiological processes. As a major element of the brain ECM, HA is abundant in white-matter tracts [42]. These are the preferred migration routes for neural stem cells, eg, after transplantation into the injured rodent brain [43–45], and similarly these tracts constitute dissemination paths for tumour cells in glioma [11, 14, 46]. HA is also present in the neurogenic niche and important for stem cell maintenance [47]. Under pathological conditions, such as injury, inflammation, and repair, it undergoes regulations. The HA polymer becomes degraded by a series of enzyme reactions, thus generating HA with a range of molecular weights that are involved in various biological functions [37]. Low and intermediate molecular-weight HA has distinct biological functions as compared to native high molecular-weight HA, for example stimulating gene expression in macrophages, endothelial cells, and certain epithelial cells as well as scar formation [48]. In glioma, low molecular-weight HS oligomers have been shown to act

as an inhibitor of hyaluronan-dependent release of putative effector molecules from tumour cells [49].

HA interacts with several receptors [50] out of which CD44 and RAHM (receptor for hyaluronic acid-mediated motility) affect cell growth and motility, and thereby has the ability to mediate primary tumour cell invasion and migration [51, 52]. CD44 is the principal cell surface receptor for HA, and HA-CD44 interactions play a crucial role in eg development and inflammation, tumour growth, and metastasis [53, 54]. Signalling through the intracellular part of CD44 was shown to affect cell adhesion and motility through interactions with cytoskeletal proteins [55, 56]. In addition, CD44 interacts with a large number of signalling molecules that promotes matrix degradation and the spread of tumour cells (reviewed in [57]). HA-independent roles for CD44 have also been proposed in cell adhesion/migration because CD44 can act as a cell-surface anchor for the ECM-degrading enzyme MMP-9 [58].

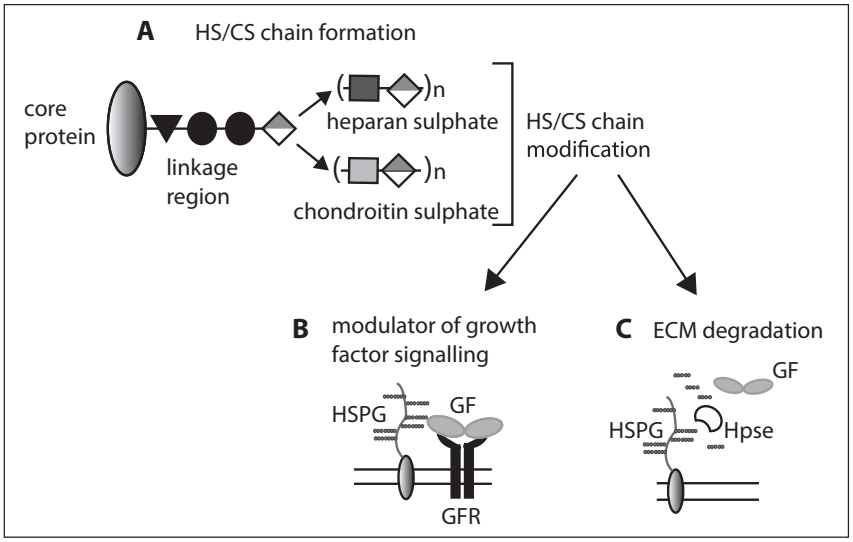

Figure 1. (A) Schematic representation of heparan sulphate (HS) proteoglycan and chondroitin sulphate (CS) proteoglycan biosynthesis and chain modifications. **(B)** Heparan sulphate proteoglycans (HSPG) act as co-receptors for growth factor (GF) signalling via growth factor receptors (GFR), thereby promoting cell proliferation, angiogenesis, and migration. **(C)** Degradation of HSPG by heparanase (Hpse) contributes to ECM degradation and release of HS-bound growth factors.

Heparan Sulphate

Heparan sulphate proteoglycans (HSPG) are found either at the cell surface (syndecans and glypicans) or secreted (eg, perlecan and agrin) [59]. HSPGs are main components of the ECM where they interact with a large number of physiologically important macromolecules, thereby influencing biological processes [60]. HSPGs modulate growth factor activities, regulating interactions between ligand and receptor [61], and during CNS development, morphogen gradients can be maintained by HSPGs [62] (Figure 1). Its unique molecular design is composed of clusters of N- and O-sulphated sugar residues, separated by regions of low sulphation, which determines specific protein-binding properties [59]. In the adult brain, HS is associated with neural stem cell niches [63], where it may be involved in regulating neural stem cell maintenance. Recently, levels of 6O-sulphation of HS have also been linked to injury response as increased sulphation promoted glial scarring [64].

Mouse gene knockout experiments have shown the vital role of HSPGs in development and homeostasis [65]. Studies on neural differentiation have, to a large extent, been performed on *in vitro* differentiated ES cells to neural progenitors due to early embryonic lethal phenotypes of mice with deletions in crucial HS chain modification enzymes [66]. Our recent publication showed that N-sulphation enzymes in HS biosynthesis are indispensible for neural differentiation and that the ratio between HS and FGF was the crucial factor determining neural differentiation [67].

It has long been known that HSPGs are involved in the progression of various cancers [68] including glioma, where the levels of HSPGs are higher than in the normal brain [69]. Syndecan-1 [70] and glypican-1 [71] are examples of HSPG core proteins with increased expression in glioma cells compared to non-neoplastic cells. Su et al [71] found that glioma cell-associated HSPG was more capable of stimulating FGF-2 signalling than HSPG from normal cells, further supporting the role for HS-mediated growth factor signalling in glioma. There are several receptor tyrosine kinase pathways that can be modulated by altered HS amounts and composition. The extracellular sulphatase Sulf-2, which controls 6O-sulphation, thereby regulating HSPG interactions with eg PDGF receptors, has recently been shown to be highly expressed in glioblastoma and demonstrated to increase tumour size [72]. Increased Sulf-2 expression was most prominent in the proneural subclass that is associated with increased PDGF receptor activity, and the PDGF alpha receptor pathway was particularly influenced by altered Sulf-2 levels although IGF1 receptor and EPHA2 stimulation were affected as well.

A systematic analysis of proteoglycan expression of glioblastomas included in The Cancer Genome Atlas [73] showed that several of the proteoglycans as well as their biosynthetic and degradation enzymes were differently regulated in GBM compared with the normal brain [41]. The authors found by means of database mining that biosynthetic enzymes were mostly down-regulated, except for HS3ST3a1, as previously reported [71]. Sulphation of HS is critical for its function as a co-receptor for various growth factor receptors, and therefore the increase in 3O-sulphation (by HS3ST3a1) and decreased 6O-sulphation (by increased Sulf2 [72] may be specific for GBM [41].

Heparanase, the major HS-degrading enzyme, was found to be up-regulated in TCGA GBM patients [41]. There are some reports implicating heparanase in glioma. In a study using human glioma cell line U87, heparanase over-expression led to increased motility and invasion. Larger tumours were found after xenografting of glioma cells with elevated heparanase, whereas at further increased levels, tumour growth was inhib-

ited [74]. Another study reports that heparanase was not expressed in human GBM and that U87 glioma cells lost their heparanase expression upon intracranial grafting to form tumours [75]. In contrast, Hong et al [76] found that mRNA levels of heparanase were elevated in glioma of various WHO grades relative to normal human brain, and that heparanase-expressing tumour cells *in vivo* had elevated proliferation. Similarly, U251 glioma cells over-expressing heparanase have increased proliferation, migration, and colony formation, accompanied by increased AKT phosphorylation [77]. Recently, heparanase has been shown to be a target gene for TGL1 [78], an alternatively spliced form of the GLI1 transcription factor, which causes migration and invasion of glioblastoma [79].

Chondroitin Sulphate

Chondroitin sulphate occurs as sulphated GAGs covalently attached to a core protein on cell surfaces and within extra-/pericellular matrices [80]. The synthesis of CSPG starts in a similar way as the HSPGs, in the endoplasmic reticulum/Golgi compartment with the synthesis of the GAG-protein linkage region. Like the HS, CS moieties vary considerably in their size and number of chains per protein core and in the position and degree of sulphation. A wide variety of biological functions have been attributed to CSPGs including cytokinesis, morphogenesis, and neuronal plasticity. It is also described to play an important role in pathological processes like brain injury, infections, and cancer metastasis [27].

As is the case with HSPGs, CSPGs show high expression in neurogenic niches of the brain, where they play important roles in regulating growth factor signalling [81]. Furthermore, the presence of CSPGs on neural progenitors is correlated to their ability to respond to FGF-supported proliferation and EGF-induced migration [82]. Neuronal migration and formation of neurites are believed to depend on over-sulphated CS [83]. Studies of knockout mice deficient in CS sulphotransferases confirmed that these enzymes are needed for radial migration of cortical neurons [84].

It has long been known that CSPG4, also referred to as NG2, is a marker of oligodendrocyte progenitor cells (OPC) [85], and that it promotes proliferation and migration by functioning as a co-receptor for growth factor signalling. Both PDGF-AA and FGF2 bind NG2 core protein [86], and a large number of studies have delineated the role for these interactions in oligodendrocyte biology [87]. Moreover, NG2 was also found to regulate EGFR signalling in OPCs and to contribute to their polarity [88]. Apart from OPCs, NG2 is also expressed on pericytes in the brain, and thereby involved in maintaining the integrity of the blood-brain barrier [89]. After injury of the CNS, CSPGs are the main inhibitory molecules of the glial scar [90], thereby creating barriers to prevent injury/inflammation to spread. In doing so, CSPGs also block axonal re-growth into the injured area, thereby counteracting regenerative processes. CSPGs thus make up important molecular boundaries in the brain, preventing cell movement.

A role for CSPGs in malignant progression of glioma was suggested several years ago [91] and followed by many other studies [92]. In many cancers, the expression of versican is increased [93]. Furthermore, NG2 knockout mice exhibit reduced tumour angiogenesis [94] and CSPG levels also regulate immune cell infiltration in tumours [95]. In an experimental model of glioblastoma, GBM over-expressing NG2 gave rise to larger, more vascularised tumours as compared to tumours from NG2-negative cells [96]. Patients with high NG2 levels had a shorter survival time, suggesting NG2 as a prognostic predictor. These authors showed that elevated NG2 expression was correlated to increased resistance to radiation, and an induction of scavenging enzymes was proposed as one of the mechanisms behind this observation [97]. Furthermore, human GBM-derived NG2-positive tumour cells generated more aggressive tumours than the NG2-negative population [98]. An oncolytic virus was reported to be more efficient when CS was enzymatically removed and the tumours became smaller, without signs of increased tumour spread [99]. In addition, expression of the core proteins and the biosynthetic enzymes for CSPGs were found to be predominantly over-expressed in human glioblastoma based on the analysis of the TCGA database [41]. Taken together, these studies suggest CSPGs as targets in glioblastoma therapy.

The notion that CSPGs promote invasiveness of brain tumours despite their function to form barriers in the normal and injured brain may seem somewhat contradictory. For example, the lectican family of CSPGs is the most abundant CSPG in the adult brain where it provides a structural and functional link from the cell into the matrix, which is mainly composed of HA [100]. Lectican deposition has thus been shown to be the major inhibitory molecules for axonal outgrowth both *in vitro* and *in vivo* [27]. Nonetheless, the lectican family core proteins are highly up-regulated in GBM with the exception of neurocan [41]. This potential inconsistency was recently discussed by Silver et al [29]. In their study, the authors showed that CSPG borders were associated with contained, non-invasive tumours, and that glycosylated CSPGs were absent from diffusely infiltrating GBM. They propose that the degree of glycosylation of CSPGs is inversely correlated to the invasiveness of glioblastoma. This notion is supported by a previous study in which a less glycosylated form of the CSPG brevican was associated with human glioma [101]. Thus, the side chain modifications of CS would be of importance, not only the expression levels of the CSPG core protein.

Concluding Remarks

In conclusion, we have discussed the effector roles for glycosaminoglycans, including hyaluronan and the proteoglycans HSPG and CSPG in glioblastoma growth and invasion. These molecules and the enzymes regulating their synthesis and degradation are abundant and differently regulated in glioblastoma compared to the normal brain. They may therefore constitute new therapeutic opportunities for targeting malignant glioma, a disease characterised by diffuse infiltration and local invasion.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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