KERATAN SULPHATE IN THE TUMOUR ENVIRONMENT

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Short running head: KS and tumours

Abstract

Keratan sulphate (KS) is a bioactive glycosaminoglycan (GAG) of some complexity composed of the repeat disaccharide D-galactose β1→4 glycosidically linked to N-acetyl glucosamine. During the biosynthesis of KS a family of glycosyl transferase and sulphotransferase enzymes act sequentially and in a co-ordinated fashion to add D-galactose (D-Gal) then N-acetyl glucosamine (GlcNAc) to an GlcNAc acceptor residue at the reducing terminus of a nascent KS chain to effect chain elongation. D-Gal and GlcNAc can both undergo sulphation at C6 but this occurs more frequently on GlcNAc than D-Gal. Sulphation along the developing KS chain is not uniform and contains regions of variable length where no sulphation occurs, regions which are monosulphated mainly on GlcNAc and further regions of high sulphation where both of the repeat disaccharides are sulphated. Each of these respective regions in the KS chain can be of variable length leading to KS complexity in terms of chain length and charge localization along the KS chain. Like other GAGs it is these variably sulphated regions in KS which define its interactive properties with ligands such as growth factors, morphogens and cytokines and which determine the functional properties of tissues containing KS. Further adding to KS complexity is the identification of three different linkage structures in KS to Asparagine (N-linked) or to Threonine or Serine residues (O-linked) in proteoglycan core proteins which has allowed the categorization of KS into 3 types, namely KS-I (corneal KS, N-linked), KS-II (skeletal KS, O-linked) or KS-III (brain KS, O-linked). KS-I to III are also subject to variable addition of L-fucose and sialic acid groups. Furthermore, the GlcNAc residues of some members of the mucin-like glycoprotein family can also act as acceptor molecules for the addition of D-Gal and GlcNAc residues which can also be sulphated leading to small low sulphation glycoforms of KS. These differ from the more heavily sulphated KS chains found on proteoglycans. Like other GAGs, KS has evolved molecular recognition and information transfer properties over hundreds of millions of years of vertebrate and invertebrate evolution which equips them with cell mediatory properties in normal cellular processes and in aberrant pathological situations such as in tumourogenesis. Two KS-proteoglycans in particular, podocalyxcin and lumican are cell membrane, intracellular or stromal tissue associated components with roles in the promotion or regulation of tumour development, mucin-like KS-glycoproteins may also contribute to tumourogenesis. A greater understanding of the biology of KS may allow better methodology to be developed to more effectively combat tumourogenic processes.

Key words: Keratan sulphate; Sulphation motifs; tumour marker; podocalyxcin; phosphacan; keratocan; KS mucin glycoproteins; KS-Antibodies, 5-D-4, 1-B-4, MZ-15, 4C4, R-10G, D9B1; SV2 proteoglycan; aggrecan; astrocytomas.

Please note that the KS antibodies refer to in this chapter are directed to epitopes in the glycosaminoglycan keratan sulphate and should not be confused with anti-aminoacyl-tRNA synthetase (ARS) antibodies which have also been referred to as KS antibodies [1] or the anti-cyclin D1/D2 antibody which is also referred to as 5-D-4 [2].

1. Introduction

Glycosaminoglycans (GAGs) have evolved over hundreds of millions of years of vertebrate and invertebrate evolution through positive evolutionary selection pressures which have resulted in GAGs being chosen which have an ability to participate in a diverse range of essential physiological processes [3,4]. GAGs are sophisticated biodiverse components of the glycocalyx surrounding all cells which convey important molecular recognition and structural information important in cellular regulation and tissue homeostasis [5-10]. While GAGs are composed of regular repeat disaccharides it is the non-uniform sulphation patterns along the GAG backbone which have important extracellular matrix and cell regulatory properties and these are the functional determinants on GAGs which equip them with interactive properties with extracellular matrix (ECM) components, growth factors, morphogens and cytokines which regulate tissue development/remodelling and the maintenance of tissue homeostasis in health and disease [5,11-14]. Significant alterations in GAG distributions and composition have been noted in a number of tumours, these are of diagnostic value and tumour secretions containing these GAGs have proven useful as biomarkers of the pathological status of tissues and the degree of tumour development or regression following therapeutic intervention [15,16].

2. Keratan Sulphate Structure

Keratan sulphate (KS) is a GAG which has a widespread distribution in connective tissues [17,18]. KS is composed of the β1-4 glycosidically linked repeat disaccharide Gal-GlcNAc which are sulphated at C6 either individually or collectively leading to regions of mono or disulphation in the KS chain, regions of non-sulphation have also been identified referred to as (poly) N-acetyl lactosamine regions in the KS chain although a number of proteins also contain lactosamine (Figure 1). The linkage region at the reducing terminus of the KS chain to proteoglycan (PG) core protein acts an acceptor molecule for saccharide attachment. During KS biosynthesis chain elongation and sulphation are co-ordinated events and elongation of the KS chain occurs by step-wise addition of GlcNAc or Gal co-ordinated with sulphation of these moieties [17,18]. Several glycosyl transferases and sulphotransferases are involved in KS biosynthesis, these are shown in Figure 2 reproduced from KEGG KS biosynthesis reference data (Map 00533) [http://www.kegg.jp/kegg-bin/show_pathway?map00533]. GlcNAc 6-O-sulphotransferase acts only on terminal non-reducing terminal GlcNAc residues on the nascent KS chain. Failure to add sulphate to a terminal GlcNAc residue may result in a disaccharide unit devoid of sulphate or having one sulphate group located on the GlcNAc residue only, D-Gal sulphotransferase only acts on a KS disaccharide if the GlcNAc is first sulphated giving rise to a disulphated disaccharide thus heterogeneous

distributions of mono- or disulphation or non-sulphation can also occur along a given KS chain. GlcNAc normally undergoes sulphation more frequently than Gal in the KS disaccharide. Like all GAGs the sulphation status of KS defines its functional properties.

2.1 Keratan sulphate biodiversity

KS has been categorised into three types on the basis of differences in the structures of the linkage region they utilise to attach to PG core proteins and in their internal structural organisation (Figure 1). KS-I was the first form of KS identified, cornea is the richest tissue source of this GAG leading to its historical naming as corneal KS [19], however this form of KS also decorates a number of PGs with a widespread tissue distribution in a range of tissues other than the cornea thus its naming is a historical misnomer. KS-II or skeletal KS exclusively decorates the major cartilage PG aggrecan. A further form of KS has been identified in brain (KS-III) which is rare in non-neural tissues but occurs in ~30% of all brain glycoproteins and PGs (Figure 1).

Corneal KS (KS-I) is attached to Asn in PG core proteins via a complex-type *N*-linked branched oligosaccharide, whereas in cartilage, KS-II is *O*-linked via GlcNAc to Ser or Thr residues via a mucin core-2 structure [17]. Brain KS-III uses a third type of linkage to protein via mannose *O*-linked to Ser or Threonine [20]. These linkage oligosaccharides are shown in Figure 1. KS is a

heterogeneous GAG and exhibits both variation in chain length and in sulphation along the KS chain. Five regions can be identified in KS-I, (i) the non reducing terminal end-capped region, (ii) di-sulphated region, (iii) monosulphated region, (iv) non sulphated lactosamine region, (v) the linkage region to PG core protein. Equivalent regions in KS-II and KS-III also occur but the lengths of individual regions and sulphation patterns may differ leading to a considerable level of size and charge heterogeneity in KS. Furthermore, the size distribution and degree of sulphation of KS chains increases with tissue development and maturation and the age of the connective tissues and its pathological status. High charge density KS has been observed associated with a number of tumours thus its analysis can be of diagnostic value.

In porcine corneal KS, the C-6 branch of the linkage oligosaccharide is extended but the C-3 branch is terminated by a single lactosamine capped by sialic acid [21]. Sulphation in porcine corneal KS is distributed non-randomly, two non-sulphated lactosamine disaccharides are present nearest the reducing terminus but 10–12 sulphated GlcNAc disaccharides are found on the more distal part of the chain. The non-reducing terminal region is of variable length and contains disulphated GlcNAc and Gal disaccharides sulphated at C6 [22-39]. Corneal KS displays a single branch in the linker oligosaccharide, extension of the other branch in the biantennary oligosaccharide is also occasionally possible [reviewed in [18]. The non-reducing

ends of KS-I chains are terminated with neuraminic acid, βGalNAc, or αGal end-capping structures [39,40]. Despite its name, KS-I is found in tissues other than the cornea such as in cartilage N-linked KS chains occur on fibromodulin, lumican, PRELP (prolargin), keratocan and osteoadherin [22,25,38]. Aggrecan contains 2–3 *N*-linked KS chains in addition to 20 or more *O*-linked KS-II chains in the KS rich region adjacent to CS substituted regions on the aggrecan core protein [24]. A few KS chains are also interspersed in the CS1 and CS-2 regions in aggrecan these differ from the KS chains of the KS rich region in that they can be heavily modified by fucosylation and sialylation making them immunologically distinguishable. The amino terminal G1 and G2 globular domains of aggrecan and the interglobular domain (IGD) between these contain a few small KS chains however these are of low sulphation and can be *N*- or *O*-linked. Some of these KS chains in G1 obscure T cell epitopes which otherwise make the G1 domain a potent arthritogen in inflammatory arthritis. KS chains within the IGD potentiate the action of ADAMTS-4 and ADAMTS-5 which cleave in the IGD and elsewhere in the aggrecan core protein. These enzymes are important for aggrecan turnover however excessive ADAMTS activity results in cartilage degeneration and pathological tissue changes in OA and RA. PZP3 zona pellucida glycoprotein carries KS-I chains however these differ from the KS-I chains found in cornea [36]. Similarly, KS-I in fibromodulin is relatively short (8–9 disaccharides), more highly sulphated [34] and lacks the characteristic domain structure of corneal KS and

its non-reducing terminal end-capping saccharides resemble those found in cartilage KS-II rather than corneal KS-I [34], thus such capping structures are tissue-specific rather than KS type specific. KS-II in the KS rich region of aggrecan, contains 5–11 highly sulphated disaccharides, interrupted only occasionally by mono-sulphated KS and its non-reducing terminal region is capped by neuraminic acid attached at C3 or C6 to terminal GlcNAc. Furthermore fucose is attached to C3 of sulphated GlcNAc throughout the KS chain but not within four residues of its non-reducing terminus [26]. KS-II from non-weight bearing tracheal cartilage is not fucosylated, and carries only (2→3) linked neuraminic acids at the non-reducing terminus [27,35].

2.2 Keratan sulphate antibodies

Monoclonal antibodies to KS (Table I) react with extracts from most mammalian tissues, at least sixteen ECM PGs substituted with KS and several intracellular and cell associated KS-PGs have been identified [reviewed in [17,18]]. All GAGs other than KS contain at least one negative charge per disaccharide, the lack of uronic acid in KS and variable sulphation of its lactosamine residues results in charge heterogeneity in KS [17,18]. Furthermore, a number of poly-*N*-acetyl lactosamine modified proteins exist which would be classified as KS-PGs if some of their residues were sulphated [32]. The development of MAb R10G and 1B4 allows KS-PG species of low sulphation and mucin-like proteins containing lactosamine regions containing GlcNAc

and Gal residues that are sulphated to be identified as KS-PGs (Figure 3). Formerly, antibodies such as 5D4 and MZ-15 which detect high charge density KS glycoforms were routinely used in this research area however these do not detect such low sulphation forms of KS thus a new aspect of the biology of KS-PGs of low sulphation is now emerging [41-43].

2.3 Keratan Sulphate complexity in healthy and diseased tissues

KS and its specific roles in tumours, spinal cord and brain

Analysis of GAGs associated with normal and tumour tissues and tumour cells [15,44-54] and their secretions [48,55] has identified the glycan signatures of pathologic tumourogenic tissues and shown these are of diagnostic and prognostic value [15,56]. Changes in the PG compositions associated with tumour masses have also been identified [57,58]. KS is a prominent component of many tumours including carcinomas of the genital tract [56], prostatic secretory cells [44], brain and ovarian tumours [53], papillary carcinomas of the human thyroid gland [59] and granular cell tumours [45]. The human embryonal carcinoma marker antigen TRA-1-60 identifies a sialylated KS-PG [60]. Chondrosarcoma cells synthesise a characteristic KS-PG in long-term culture [57]. Improved methodologies have been developed for the structural characterisation of KS produced by ovarian and brain tumours [53]. KS-substituted isoforms of thyroglobulin and transferrin are uniquely elaborated in papillary thyroid carcinomas [61]. Highly sulphated KS is synthesized in

malignant astrocytic tumours [47,62], and glioblastoma [46]. Lumican is a prominent KS-PG associated with a number of tumours (Table III) and has roles in the regulation of tumour cell growth, migration and attachment to ECM components [63-66]. Another KS-PG, podocalyxcin has also been found associated with malignant astrocytic tumours [62]. Monoclonal antibody 4C4 specifically recognizes KS-PG on human embryonal carcinoma cells [67]. KS has been identified as a prominent component of pathological brain tissues. KS is produced by microglial cells in the development of amyotrophic lateral sclerosis (ALS)[68-71]. A reduction in KS levels in brain tissues accelerates the development of ALS [72] and Alzheimer's disease (AD) [73,74].

In the intact normal spinal cord, microglial cells and macrophages express the 5D4 KS epitope however astrocytes do not [75]. A focal upregulation of 5D4 reactivity occurs associated with glial scar formation following spinal cord injury apparently due to glial cell activation and an influx of macrophages to the lesion site (Figure 4). Proteoglycans are up-regulated in the spinal cord lesion site and this stabilises this structure however the KS and CS side chains of these PGs strongly inhibit nerve outgrowth and axonal regeneration [76-78]. Therapeutic administration of keratanase, chondroitinase B and chondroitinase ABC significantly improves spinal cord regeneration in experimental rat models and suggesting these as appropriate therapeutic interventions to improve recovery of human spinal

cord injury [76,79,80]. Fragmentation of aggrecan occurs in the normal intact spinal cord through the action of aggrecanase and metalloprotease enzymatic activity and the abundance of aggrecan fragments increases with spinal cord injury[81-86]. Up-regulation of ADAMTS-4, 5 in the spinal cord lesion site is associated with areas of improved repair post injury and these have been suggested to be of therapeutic value however these findings need to be carefully evaluated [84,87].

KS also has roles in the pathogenesis of ALS and in the activation and proliferation of microglial cells [69]. KS binds to Shh and regulates the differential switch from motor neuron to oligodendrocyte during spinal cord development [88]. Phosphacan containing high charge density 5D4 positive KS chains regulates the development of the mouse visual cortex [89]. KS inhibits neural regrowth [90] and directs the development of the trigeminal nerve during corneal development [91]. KS has interactive properties with a large number of nerve regulatory proteins through which it can regulate neural development through interaction with members of the Robo, Slit, Ephrin, Ephrin receptor and Semaphorin families and two further nerve growth factor receptors [92]

2.3.1 Mucin type glycoproteins:

The role of KS substitution in tumour development

Membrane bound and secreted mucin type glycoproteins contain GalNAc, GlcNAc, Gal, Fuc, N-acetyl neuraminic acid attached to their core proteins through *O*-linkage to Ser and Thr residues on their tandem repeat domains leading to a bottle brush type structure reminiscent of PGs such as aggrecan (Table II). A family of sulphotransferases can sulphate the GlcNAc and Gal residues in mucins thus some mucins carry KS chains [93], MUC1, MUC4, and MUC16 synthesised by normal cultured bronchial epithelial cells bear 5D4 positive KS [94]. MUC16 is the largest transmembrane mucin with a molecular weight ranging from 2.5 to 5 MDa. MUC16 lubricates and protects the mucosal epithelium of the upper respiratory tract, ocular surface, mesothelial pleural, peritoneal and lining tissues of the male and female reproductive organs. MUC16 contains extracellular and transmembrane domains as well as a cytoplasmic domain which interacts with the ERM cytoskeletal actin-binding proteins ezrin, radixin and moesin. MUC16 is also associated with tumour cells, its extracellular domain is cleaved from ovarian cancer cell surfaces into the blood stream where it is useful as a tumour biomarker through identification of a peptide epitope (CA125) which also promotes cancer cell proliferation [41,42,95]. Cultured human tracheobronchial epithelial cells synthesise 5D4 KS positive MUC1, MUC4, and MUC16 tethered to cilia and microcilia however no PGs have been detected in the epithelial glycocalyx (Figure 5). KS on the mucin associated cilia and ciliary

plumes provide a protective layer extending as far as 100 μm from the epithelial cell surface [94].

The sulphated glycans on epithelial mucins effect cell adhesion and regulate the biosynthesis, half-life and biological roles of glycoproteins controlling lymphocyte homing and inflammation in the epithelial mucosa. Two sulphotransferase families transfer sulphate from 3-phosphoadenosine 5-phosphosulphate (PAPS) to C3 of Gal (Gal3ST) or C6 of GlcNAc (GlcNAc6ST) in mucins. The ubiquitous mucin core 1Gal3ST, acts on O-linked Galβ1–3GalNAc α-R in most tissues, with high activity levels in rat colonic mucosa and is also upregulated in inflamed cartilage, intestine and lung tissues in tumour development. KS has been immunolocalised to the cell associated mucins MUC1, MUC4 and MUC16 [94]. O-glycan mucin core structures 1–4 and 6 act as potential substrates for sulphotransferases [96-100], sulphation on Gal and GlcNAc residues of N-acetyl lactosamine occurs at C3 of Gal and C6 of GlcNAc [93]. Human mammary epithelial cells synthesise PGs containing *O*-linked sulphated GlcNAc attached to core 2 structures [101] (Table 2). MUC-1 in human endometrial tissue carries 5D4 positive KS and a sialo-KS epitope recognized by Mab D9B1 [102]. These epitopes convey adhesive and anti-adhesive properties which regulate embryo implantation [102]. These KS epitopes are independently regulated in the endometrial endothelium due to hormonal control with the 5D4 epitope abundant on the luminal epithelial

surface until implantation, thereafter it gradually disappears, D9B1 binding sites are retained in the luminal endometrial epithelium following implantation [103].

An endothelial mucin-like adhesion molecule (Glycam-1) binds L-Selectin through C6 sulphated GlcNAc and Gal on O-linked Sialyl Lewis^X like structures [104,105]. Sulphation of Sialyl Lewis^X structures significantly improves their L-Selectin binding properties. The sulphation motifs on mucins act as binding modules for bacteria but also protect the mucins from depolymerisation by bacterial glycosidases. Changes in mucin sulphation alter growth factor interactions, leucocyte homing and adhesion under inflammatory conditions [106]. In monocytes TNFα induces expression of 6-sulfo N-acetyl lactosamine (LacNAc)/Lewis X epitopes on *N*-and *O*-linked cell surface glycans altering their migratory and adhesive properties [106]. Cell surface and secreted mucins in ovarian cystadenoma [107] or human bronchial mucins [33,108] also carry such sulphated Lewis X L-selectin ligands which promote leucocyte attachment to the endothelium [109-111]. The sulphate content of mucins is decreased in colon cancer and in ulcerative colitis [112-115] due to degradative effects on mucins by bacterial sulphatase activity [116]. A significant reduction in mucin sulphation has also been observed in colorectal adenoma cells as they progress to a cancerous state. This decrease is due to decreased core 1 Gal3ST and GlcNAc6ST expression [117]. Lower Gal3ST activity

is also a feature of colon cancer [118-120] and breast cancer cells compared to normal mammary cells [96]. The mucin core structures biosynthesized and their associated modifications in cancer [120] influence the amount of mucin sulphation. Alterations in the expression patterns of sulphated mucins and sulphotransferases in inflammatory diseases and cancer alters the distribution and density of mucin sulphation motifs and adversely influencing disease progression [93].

3. Brain contains a number of multifunctional KS-PGs

A number of diverse KS substituted PGs have been identified in the brain (Figure 6). Aggrecan is a component of perineuronal nets which surround and protect neurons and promote neuritogenesis and synaptic plasticity [121]. Podocalyxcin is a transmembrane KS-PG with cell signalling capability widely distributed in neurons. In embryonic tissue podocalyxcin isolated from pluripotent neuroprogenitor cells contains low sulphation KS chains and has been used as an antigen for the production of antibodies which identify these low sulphation KS glycoforms. However in pathological neural tissues tumour cells express podocalyxcin decorated with high charge density KS glycoforms identified by antibodies 5D4, and MZ-15 [122,123] and these may also be of diagnostic value (Figure 6).

Podocalyxcin is an anti-adhesive transmembrane neural KS-

polysialylated- proteoglycan/glycoprotein with essential roles to play in neural development [124,125] and is also a marker of human embryonic and induced pluripotent stem cells [126]. Podocalyxcin is upregulated in glioblastoma formation and in astrocytomas [46,47,62,127-130], and has been developed as a prognostic factor for various cancers [131,132]. The sulphation status of the KS chains on podocalyxcin on normal embryonic cells and tumour cells differ with the former expressing a low sulphation KS detected by MAb R-10G [133-135] while tumour cells produce a high sulphation KS chain [62] detected by antibodies such as 5-D-4, MZ-15 or 4C4 [67,122,123].

Two cytosolic adaptor proteins, Na+/H+-Exchanger Regulatory Factor 2 (NHERF2) and Ezrin, interact with the cytoplasmic tail of podocalyxcin in kidney and similar interactions with cytoskeletal components also occur in neural tissues exerting regulatory effects on cell signaling and downline effects on neural behavior during the development and repair of the CNS/PNS[136,137]. Neural migration and axonal guidance are governed by cues from many ECM molecules (Netrins, Semaphorins) which exert either attractive or repulsive cues. Podocalyxcin is not essential for neural migration to occur but can modulate this process [121]. Cell-cell contact and adhesion to the ECM contribute to neural assembly processes. Adhesion molecules such as NCAM and L1 have important roles to play in axonal growth, neural migration and synapse formation. Co-ordination of ECM signals is essential

in such developmental processes. Podocalyxcin has essential roles to play in neuritogenesis and synaptogenesis [138-140]. Podocalyxcin co-localises with synapsin and synaptophysin in synapse vesicle formations [124]. Synaptophysin is a major synaptic vesicle protein which co-ordinates the endocytosis of synaptic vesicles during neural stimulation [141], synapsin tethers synaptic vesicles to cytoskeletal components preventing premature vesicle release into the synaptic gap co-ordinating neurotransmitter release from the synaptic vesicles [142-145].

4. SLRPs and their roles in cell migration, proliferation and regulation of growth factors and inflammatory cytokines in a diverse range of tissues in health and disease.

The SLRPs have multiple functional roles in soft connective tissue ECMs where they regulate collagen fibrillogenesis and regulate growth factor and inflammatory cytokine activities (Figure 7). Not only do the SLRPS maintain the integrity of tissues but their levels are elevated in OA and RA [146] and in animal models of OA [147]. Lumican binds to C1q and regulates complement activation contributing to innate immune protection [148] and may also contribute to the OA/RA pathogenic processes. Specific SLRP members such as lumican regulate cell migration and proliferation and have roles to play in tumour growth, local invasion, extravasation and invasion of remote anatomic sites [65].

Lumican plays essential roles in the regulation of collagen fibrillogenesis in different ECMs however there is considerable redundancy in the SLRPs. Lumican is also expressed in the developing bone matrix. Real-time PCR OF MC3T3-E1 cell cultures showed that the expression of lumican increased as the osteoblast culture differentiated, suggesting a role for lumican in the regulation of collagen fibrillogenesis in bone matrices [149]. During early embryonic murine development (E11 to E13), lumican is mainly expressed in the cartilaginous rudiments however by E14 to E16 lumican expression is more prominent in the developing bone. Lumican is secreted by differentiating and mature osteoblasts and can be used as a marker to distinguish proliferating pre-osteoblasts from the differentiating osteoblasts [149]. Lumican, keratocan and osteoadherin are all class II SLRPs [150] which interact with TGF-β, BMP4, WISP-1 (Wnt1-inducible secreted protein-1), von Willebrand factor, PDGF, TNF-α, and IGF-I forming growth factor concentration gradients controlling their bioavailability to cells and pericellular interactions they participate in with cell-surface receptors, modulating cell-ECM interactions which modulate tissue development and homeostasis [150]. Osteoadherin (osteomodulin) is a 49,116-Da protein containing 11 leucine-rich repeats (LRRs), 3-4 tyrosine sulphate residues at the N-terminus , and six potential glycosylation sites for N-linked KS chains within the LRR region. Osteoadherin shows 42% sequence homology to

keratocan and 37–38% identity to fibromodulin, lumican, and PRELP [38]. Osteoadherin promotes $\alpha_v\beta_3$- integrin mediated cell binding. Osteoadherin has been isolated as a minor, leucine- and aspartic acid-rich KS-PG found in the mineralized matrix of bone [151]. Osteoadherin is a relatively acidic protein which binds to hydroxyapatite and to osteoblasts through $\alpha_v\beta_3$- integrin and has been immunolocalised to pre-dentin during tooth formation [152].

5. Lumican specific roles in the regulation of tumour development

Lumican is a class II SLRP which bears significant levels of homology with other class II SLRPs such as keratocan, fibromodulin, lumican, and PRELP. Lumican is the only SLRP which occurs with such a high frequency in tumourogenic tissues leading to the proposal of lumican as a tumour cell marker.

SLRPs organize the cartilaginous and many other soft connective tissue ECMs where they have functional roles to play in tissue development, remodelling and in pathogical changes in these tissues [146]. OA is a progressive degenerative condition affecting the articular cartilage, meniscus, synovium, subchondral bone and infrapatellar fat pad in the knee-joint [153,154]. With the development of OA, PGs in these tissues undergo proteolytic degradation and some of the fragments so generated have been suggested as potential biomarkers of this disease process. Characteristic fragmented forms of the

CS/DS substituted PGs aggrecan, decorin and biglycan also occur in OA. Fibromodulin and Lumican are structurally homologous sharing 47% identity in their primary structures and both can have 4 small N-linked KS chains [155,156]. Like all class II SLRPs fibromodulin and lumican contain 11 LRRs which facilitate their interactions with other ECM components including type I and type II collagen which regulates fibril spacing and the fibrillogenesis process. Lumican regulates the regularly orthogonally spaced fine collagen fibrillar arrangements in the cornea essential for optical clarity [157-163]. Fibromodulin is more prominent in the limbus and sclera where it stabilises large collagen fibre assembly which mechanically support the eye-ball [159,164,165]. Fibromodulin has N-linked KS attachment sites on Asn residues at positions 127, 166, 201,291, and 341 in the core protein although only four of these sites are occupied by KS at any one time. Lumican also contains four N-linked KS chains located within the central LRR region at Asn 88, 127, 160, and 252. In addition, both of these SLRPs contain N-terminal sulfated tyrosine clusters, with fibromodulin containing up to nine of these residues and lumican two [148,166], this localization of charge facilitates interactions with growth factors in a similar manner to HS interactions with growth factors.

Despite this similarity in structural form ADAMTS-4, ADAMTS-5 [167], MMP-2,-3,-13 and -14 variably degrade fibromodulin and lumican during the etiopathogenesis of OA [168], releasing intact or fragmented forms of

fibromodulin or lumican from articular cartilage, meniscus and other joint tissues. These SLRP fragments act as DAMPs activating TLR-2 and -4 initiating innate inflammation, and pain pathways [169,170]. Lumican also augments LPS signaling through cell surface CD14, a bacterial lipopolysaccharide co-receptor which interacts with TLRs leading to NF◎B activation, cytokine secretion and an inflammatory response [170]. As already noted despite similarities in structure, fibromodulin and lumican display differential susceptibilities to degradation by MMPs and ADAMTS-4 and -5. Thus while fibromodulin is susceptible to degradation, lumican is far less susceptible. This may be due to lumicans ability to act as an MMP-inhibitor [171]. Lumican binds to and completely inactivates MMP-14 activity in B16F1 melanoma cells [171] inhibiting cell migration, angiogenesis, and cell-ECM interactions that normally promote tumour progression [172,173]. Lumican contains an MMP inhibitory peptide module in LRR-9 named Lumcorin [170]. MT1-MMP cleaves lumican abrogating this suppressive activity in tumour cells [174].

6. SLRPs and cancer

Specific roles of lumican in tumour cell regulation

The tumour microenvironment decisively controls cancer development by establishing a complex interplay between cancer cells and their surrounding stromal components which directs disease progression [175]. The

tumour stroma is composed of collagens, PGs, structural glycoproteins and cell adhesive proteins. Lumican prevents invasion of the ECM by tumour cells through intrinsic mechanisms which down-regulate cell signalling processes that would otherwise promote cancer cell proliferation [176]. SLRPs structurally organize the ECM [177,178]and regulate tumour cell proliferation through the regulation of angiogenic processes that are required for tumour development and cellular migratory processes that are also an intrinsic requirement for the establishment of tumour cell masses at remote sites. Lumican is associated with clinical outcome in cancer and appears tumour specific [179]. Lumican specifically inactivates MMP-14, through which it suppresses ECM remodeling, angiogenesis and cellular migration which all contribute to an inhibition of tumourogenesis [65,66,170,180-184].

As seen in Table III, lumican is associated with a diverse range of cancer types and plays many functional roles in the affected tissues however the role of lumican in cancer varies with tumour type. Lumican is expressed and secreted by human melanoma cells but not by normal melanocytes [185]. Lumican binds to α2β1 integrin and inhibits melanoma cell adhesion [186]. Melanoma cell migration is also blocked by inhibiting MMP-14 [173], lumcorin a peptide derived from lumicans ninth LRR repeat is a potent MMP inhibitory peptide. Lumcorin inhibits tumour cell growth [187] and migration [184] through alterations in focal adhesion complexes [180]. Actin cytoskeletal organisation

has also been shown to be disrupted by lumican binding to α2β1 integrin in A375 melanoma tumour cells [183] and it also inhibits proliferation of B16F1 melanoma cells and lung metastasis [181].

Lumican also inhibits pancreatic tumour cell growth [188]. Lumican is expressed in alpha cells of pancreatic islets and pancreatic cancer cells [189]. Lumican stimulates growth but inhibits replication and invasion by human pancreatic cancer cells [190,191] and in pancreatic ductal adenocarcinoma [192]. Lumican expression is also up-regulated in lung adenocarcinoma and squamous cell carcinoma where it inhibits cell migration and cellular proliferation [193,194] but is down-regulated in giant cell bone tumours [195].

Overexpression of lumican upregulates gelsolin and filamentous actin reorganization [196] and is associated with a good outcome in Stage II, III colon carcinoma [179]. However lumican expression in advanced colorectal cancer with nodal metastasis correlates with a poor prognosis [197,198]. In osteosarcoma lumican regulates tumour cell adhesion by modulating TGFβ2 activity [199] and is positively correlated with differentiation but negatively with the growth of human osteosarcoma cells [200]. In prostate cancer an increase in lumican expression has been observed in the stromal tissue surrounding prostate primary tumours. In-vitro experiments showed that lumican inhibited the migration and invasion of

metastatic prostate cancer cells isolated from lymph node, bone and brain. A significant increase in prostate cancer cell invasion has been observed in the peritoneum of lumican knockout mice, demonstrating the inhibitory role lumican normally plays in the ECM preventing prostate cancer invasion [201].

Lumican significantly attenuates breast tumour cell functional properties, including proliferation, migration and invasion in-vitro. Lumican also down-regulates estrogen receptor α/β expression in breast cancer cells suppressing the expression of major matrix effector molecules such as MMPs and EGFR which normally promote breast cancer progression [202]. Low lumican levels is associated with a worse prognosis in lymph node-negative invasive breast carcinomas [203].

Endometrial cancer is the most frequent type of malignant gynecological tumour in the Western world with ~40,000 cases reported annually [204]. Lumican staining is more intense in endometroid-type endometrial cancer than in endometrial intraepithelial neoplasia although the functional roles of lumican in these tissues remains to be fully determined [205].

Lumican is a cytoplasmic and pericellular component of neuroendocrine tumours including carcinoid tumours and neuroendocrine cell carcinomas

and their associated stromal tissues. Lumican is observed in the rough endoplasmic reticulum and neuroendocrine granules in neuroendocrine tumours as well as the interspaces between collagen fibers in stromal tissues and occurs in carcinoid tumours with a higher frequency than in neuroendocrine cell carcinomas [206]. High expression levels of lumican in these tissues is believed to explain the slow growth rates of such tumours. Schwannoma-like salivary pleomorphic adenomas are rare but are associated with chondroid tissue formation with the ectopic chondrogenesis driven by BMP-2. Pleomorphic adenomas are the most common form of salivary gland tumours. Lumican is predominantly found in the hyaline (100%) and fibrous regions (89.4%) and in chondroid masses in salivary pleomorphic adenomas [207].

Lumican is expressed in uterine cervical squamous cell carcinoma particularly at the periphery of cancer cell nests and by fibroblasts in proximity to these tumour cell masses but is not expressed by normal squamous or ductal cells close to these cancer cells [208]. The role of lumican in these tumours has not been determined however elevated lumican levels at the periphery of such cancer cell nests may play regulate the growth or invasion of human cervical cancer cells [208].

7. Concluding remarks

KS is an underappreciated GAG of considerable complexity. This chapter has attempted to outline the molecular recognition and information transfer properties this biomolecule conveys to a diverse array of interactive KS-PGs and the multifunctional roles they have in cellular regulation. Not only is KS attached to an extensive array of PGs with diverse functional properties but it also decorates a number of mucin-like glycoproteins of importance in the tumour environment. The interactions KS regulates are of importance in a diverse range of physiological processes in health and disease. A greater understanding of the KS glyco-code and how it is interpreted by different cell populations will undoubtably pave the way to the elucidation of further complexities of this fascinating molecule and its participation in cellular regulation in health and disease and may be of application in repair biology.

8. Future Studies on KS

Table I. Antibodies Developed to KS Illustrate its Structural Complexity

Antibody	Epitope identified	Ref
TRA-1-60	Epitope sensitive to neuraminidase, keratanase-I/II, and endo-β-D-galactosidase. Epitope identified Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc this oligosaccharide, is expressed on podocalyxcin on pluripotent embryonic stem cells	60,209-212
TRA-1-81	Epitope resistant to neuraminidase but sensitive to endo-β-D-galactosidase, keratanase-I/II. Epitope is terminal Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc these oligosaccharides are expressed on cell surface podocalyxcin on pluripotent embryonic stem cells	60,209-212
R-10G	Low sulphation poly *N*-acetyllactosamine KS epitope	61,133,134, 213
SSEA-1¶	Cell surface glycan of murine embryonic pluripotent stem cells, epitope expressed on proteoglycan and glycoprotein core proteins and bioactive lipids	195
"i" antigen¶	Human autoantibody to a non-branched epitope in non-sulphated poly-*N*-acetyllactosamine	214-218
"I" antigen¶	Human autoantibody to a branched epitope in non-sulphated poly-*N*-acetyllactosamine regions of KS	214-218
4C4	Highly sulphated KS on embryonic tumour cell podocalyxcin	67
5D4	Hexa-sulphated KS saccharide	122,123
MZ15	Hepta and octa-saccharide KS oligosaccharides	43,123
1B4	Tetrasulphated hexasaccharide in linear KS	123
3D12/H7	Trisulphated fucosylated poly-*N*-acetyllactosamine KS chains located in the CS 1 and 2 region of aggrecan core protein	219
D9B1	A sialo-KS epitope on endometrial KS-PGs	102,220,221
6D2/B5	Fucosyl-KS epitope	222
SV2	High sulphation KS chains on SV2 PG	223,224
EFG-11	Tri KS disaccharides	225
1/14/16H9	Specific equine KS antibody	226,227
BKS-1(+)	D-GlcNAc 6-sulphate KS stub neo-epitope exposed by keratanase-I/II, endo β-D-galactosidase digestion	228

Abbreviations: TRA, Trafalgar antigen/tumour rejection antigen; SSEA. Stage specific embryonic antigen. ¶ These antibodies identify non-sulphated epitopes in poly-lactosamine regions occurring in KS

Table II *O*-glycan core Mucin type acceptor structures sulphated on Gal or GlcNAc

O-glycan acceptor	Mucin source	Sulphation position	Ref
core 1 Galβ1-3GalNAc-	Rat gastric and salivary mucins	C6 on extending GlcNAc	[229]
core 2 GlcNAcβ1-6Galβ1-3	Rat mammary adenocarcinoma	C6 on GlcNAc	[230-232]
acceptor structure unspecified	Pig gastric mucin	C6 on GlcNAc	[233]
core 3 GlcNAcβ1-3GalNAc- repeat NAcetyl lactosamine disaccharides	pig zona pellucida glycoproteins	C6 on GlcNAc in *O*- and *N*- linked glycans	[97]
Specific acceptor structure not specified	Cystic fibrosis respiratory mucins	C3 on Gal and C6 on GlcNAc on multiple complex *O*-glycans	[234-236]
core 6 GlcNAc β1-6GalNAc-	Rat bone sialoprotein	C6 on GlcNAc	[99]

Table III Lumican influences many different tumour types

Tumour type	Features affected by lumican	Ref
melanoma A375, B16F1 cells	Inhibition of MMP-14 and tumour cell attachment and proliferation.	170,173,180,184,185,187,237
pancreatic cancer	Inhibition of tumour cell growth [188]. Lumican is expressed in alpha cells of pancreatic islets and pancreatic cancer cells [189]. Lumican stimulates growth and inhibits replication and invasion of human pancreatic cancer cells [190,191] and pancreatic ductal adenocarcinoma [192].	188-192
giant cell bone tumour	Down-regulation of lumican may serve as a biomarker of metastatic and recurrent giant cell bone tumours	201
prostate cancer	Anti-tumour activity. Inhibition of the migration and invasion of lymph node, bone and brain metastatic prostate cancer cells	238
colon carcinoma	Overexpression of lumican upregulates gelsolin and filamentous actin reorganization [196] and is associated with good outcome in Stage II, III Colon carcinoma [179].	179,196
colorectal cancer	Lumican expression in advanced colorectal cancer with nodal metastasis correlates with poor prognosis.	197,198
osteocarcinoma	Regulates tumour cell adhesion by modulating TGFβ2 activity [199]. Lumican expression is positively correlated with the differentiation and negatively with the growth of human osteosarcoma [200].	64,65
breast cancer	Reduced expression of lumican is associated with poor outcome in node-negative invasive breast cancer. Lumican influences ECM organisation.	239,240
adenocarcinoma and squamous cell carcinoma of lung	Upregulation of lumican inhibits tumour cell migration, and cellular proliferation	194 193,194
carcinoid tumours,	Cytoplasmic lumican in neuroendocrine tumour cells is associated with the RER, cellular	

neuroendocrine cell carcinoma	granules and the interspaces of stromal collagen fibers. Higher cytoplasmic expression of lumican in carcinoid tumours compared to neuroendocrine carcinomas may slow the growth of the former tumour cells.	[206]
salivary pleomorphic adenomas	Lumican expression is associated with the formation of mesenchyme-like elements in salivary pleomorphic adenomas.	[207]
uterine cervical cancer	lumican protein accumulates in uterine cervical cancer cells at the periphery of cancer nests	[208]
endometrial cancer	Endometrial cancer is the most common form of malignant gynecological tumour. Lumican is strongly associated with these tumours however it's functions in such tumours still has to be determined	[204,205]

Figure legends

Figure 1. The structural heterogeneity of KS assembled from the repeat disaccharide D-Gal-GlcNAc-6-sulphate showing pertinent features of Corneal KS-I and its di-, mono-, non sulphated and linkage regions plus fucose and sialic acid end-capping structures (a) and of equivalent regions in skeletal KS-II isolated from weight bearing connective tissue (b) and KS-II from non-weight bearing connective tissue (c) and brain KS-III (d).

Figure 2.

This figure is reproduced from the KEGG KS biosynthesis reference data map (Map 00533) [http://www.kegg.jp/kegg-bin/show_pathway?map00533] which shows the major known KS biosynthetic enzymes.

Figure 3.

Putative antibody recognition sites on native undigested KS-I (a) and keratanase-I, keratanase-II and endo-β-D-galactosidase cleavage sites on the KS chain (b) which generate the neo-epitope BKS-1 (+) stub KS epitope.

Figure 4

Immunolocalization of the 5D4 positive KS epitope synthesised by microglial cells and macrophages in rat spinal cord follow spinal cord injury. The arrows indicate glial cells (G) and macrophages (M) which synthesise 5-D-4 KS. Areas of co-localization are indicated in yellow. Modified from [73] with permission under the auspices of Creative Commons Attribution 4.0 International License (CC-BY).

Figure 5.

KS localised in mucus and mucins of the mucosal surface of human tracheobronchial epithelial cell cultures visualised using Haematoxylin and Eosin (a), Alcian blue-periodic acid Schiff staining (b) or by immunolocalisation of MUC5AC, MUC5B (c) and KS (MAb 5D4)(d) using specific antibodies. Panels c and d were counterstained with DAPI to visualise cell nuclei. Note the height of the accumulated mucus layer ~100 μm, the intense staining of KS in the periciliary layer and plumes of material extending from the ciliary tips into the mucus ciliary plumes (d) while excluding the polymeric mucins in panel (c). Intracellular mucins are not apparent in these images since their fluorescence intensities did not reach the detection threshold appropriate for use in the visualisation of the strong extracellular immunolocalisations . Scale bar 20 μm. Figure reproduced from [94] with permission, , Springer Nature, Mucosal Immunology (license number 4605370414328).

Figure 6.

Structural representations of the major extracellular and cellular CNS/PNS KS-Proteoglycans. Aggrecan (a), podocalyxcin (b), RPTP-ζ (c), phosphacan (d) and SV2 proteoglycan (e). Note that the structure depicted in (a) is of human aggrecan, rat aggrecan does not have a KS-rich region. Figure

modified from [15] with permission under the auspices of Creative Commons Attribution Non-Commercial License http://creativecommons.org/licenses/by-nc/4.0/.

Figure 7.

Domain structure of KS substituted SLRP family members which are found in the CNS/PNS, and tensional and weight bearing connective tissues figure adapted from[167] with permission Elsevier, Biochim Biophys Acta (license number 4605380480747).

Figure 8.

Upregulation of podocalyxcin expression in astrocytoma in the brain. Normal brain tissue showing an absence of detectable podocalyxcin (a). Assorted views of astrocytomas and immunolocalisation of podocalyxcin (b-f). Images a-c modified from [47] with permission Elsevier, Biochemical and Biophysical Research Communications (license number 4605390803553). Images d-f modified from [62] with permission Elsevier, Biochemical and Biophysical Research Communications (license number 4605390045819).

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