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Review

Glycosylation and lectins-examples of immunesurveillance and immune evasion

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Summary. Cell surface proteins are posttranslationally modified by tightly regulated enzymes of glycosylation. Typical patterns of glycosylation may signal pathological situations to the immune system. Here, carbohydrate receptors on the surface of cells in the immune system are involved in regulation of effector cells. Moreover, some lectins are circulating in the plasma and take part in host defense. The code of carbohydrate modifications is impaired in malignant cells and yet they are not eliminated. In this review, we focus on recent experimental evidence for regulatory functions of lectins and carbohydrate derivatives in the immune system and tumours.

Key words: Carbohydrate receptors, Immune system, Tumours, Lectin regulation

Introduction

Human cell surface proteins and most serum proteins are subject to glycosylation during the process of posttranslational modification. There are two biochemically distinct routes of glycosylation, O-glycosylation, where carbohydrates are covalently linked to hydroxyl groups of amino acid side chains (serine and threonine), and N-glycosylation with amino groups in the side chain of asparagines acting as acceptors. The assembly of both oligosaccharides starts with the uptake or de novo synthesis of individual sugar units. They can be interconverted into various saccharides and subsequently undergo further modifications such as sulfatation, amination, acetylation etc. These monosaccharides enter the endoplasmatic reticulum (ER), where they are transferred directly onto the protein in the case of O-glycosylation or synthesized on a carrier protein, dolichol, for N-glycosylation. From dolichol, the basic glycan ($Glc_3Man_9GlcNAc_2$) is then transferred to the nascent protein within the ER. In the Golgi compartment the glycan is remodelled by a fine-tuned process of hydrolysis and extension of the oligosaccharides. Glucose and mannose units are removed and GlcNAc, fucose, galactose and sialic acid residues are added. This complicated process of highly orchestrated enzymes has been recently reviewed in detail by Daniels et al. (2002). However, there is an exception to this rule. Members of the family of galactose-binding proteins (galectins) are released into the extracellular space bypassing the Golgi compartment.

Glycosylation products are generally branched oligomers of individual units and thus are much more difficult to analyse than linear biopolymers such as DNA made from four different units or proteins typically made from twenty amino acids. However, recent advances in spectrometric techniques and chromatography have lead to a detailed insight into the structure of glycosylation products and their changes depending on the functional state of the cell. Moreover, deletion of enzymes involved in glycosylation have further expanded our understanding of this integral process. There are numerous events where the involvement of glycosylated proteins have been identified, such as signal transduction, development, migration, cell-cell contact, immunology. Removal of one glycosylation enzyme, Mgat1, results in an embryonic lethal phenotype with impaired left-right asymmetry, defects of the neural crest formation as well as vascularization (Metzler et al., 1994). There is an increasing number of congenital disorders of glycosylation described in humans and comprehensively discussed in a recent review by Grünewald and colleagues (Grünewald et al., 2002). All affected individuals show failure to thrive, mental retardation and further organ dysfunctions, especially of liver, kidney and muscle. Unfortunately, many of these disorders lead to a fatal infectious complication in early childhood.

The role of individual enzymes involved in carbohydrate recognition has been investigated using knockout mice. Mice deficient in the sialyl transferase ST6Gal show among other features an impaired B-cell

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proliferation (Hennet et al., 1998). Deletion of the sialyl transferase ST3Gal in mice affects the CD8 T cell compartment (Priatel et al., 2000; Demetriou et al., 2001). Other mutations such as the Mgat5$^{-/-}$ mice show autoimmune disease with increased T cell proliferation (Demetriou et al., 2001). In addition to knockout models, a variety of recent studies has revealed a critical role for sialic acids during T cell selection and maturation in the thymus (Daniels et al., 2001). All these findings show that glycans are integral constituents of effector cell functions (Rudd et al., 2001).

Carbohydrate-binding proteins are termed lectins. Originally discovered in plants more than hundred years ago, they are now being identified in virtually all animals as well. In the meantime the family of lectins has grown and the most widely accepted classification was first proposed by Drickamer and is based on structural as well as functional properties (Drickamer, 1988). Some lectins require calcium for binding their ligands and are hence called C-type lectins. Another group requires reduced thiol groups for functional integrity, which led to the term of S-type lectins. A third group, P-type lectins, is comprised of receptors binding mannose-6-phosphate. I-type lectins include the Siglec family and feature immunoglobulin-like domains. Then there is the family of calnexin-homologous proteins and lastly, so far, the hyaluronan-binding proteins. In this review we will focus on lectins that have already been described to play a role in the immune system. First, we will discuss membrane-spanning receptors and subsequently soluble lectins with essential functions in the immune system. Of course, the categorization of carbohydrate receptors into surface and soluble receptors is necessarily inaccurate, as some of the soluble lectins are not only present in plasma but also on the surface of different cell types.

Cell membrane-spanning Lectins

The largest group of lectins is the functionally heterogenous family of C-type lectins. They can be divided into lymphocyte lectins, proteoglycans, endocytotic receptors, the soluble collectins, and finally selectins. Selectins are perhaps the best characterized molecules among C-type lectins. They are type I transmembrane glycoproteins with an N-terminal C-type lectin domain, which is responsible for ligand binding. This segment is followed by an EGF-like domain and various numbers of complement regulatory domains. The transmembrane region is adjacent to a cytoplasmic domain involved in activation and signal transduction. L-selectins are expressed on blood monocytes, neutrophils and subsets of NK, T and B cells. Most strikingly, L-selectin is expressed on naïve lymphocytes, both in lymphoid organs and in the peripheral blood. When lymphocytes are activated, L-selectin is shed from the cell surface, primarily through proteolytic cleavage by metalloproteinases. L-selectin is crucial for lymphocyte rolling, an early step in the extravasation process. Several ligands for L-selectin have been identified: GlyCAM-1, CD34, MAdCAM-1, and podocalyxin-like protein. These proteins require precise glycosylation with sialic acids and undergo sulfatation for optimal binding of L-selectin. This is demonstrated by CD34, which is expressed by several cell types with different post-translational modifications, and only serves on high endothelial venules as the ligand for L-selectin. Another member of the selectin family is E-selectin. Low levels of of E-selectin are expressed on vascular endothelium, but this molecule can be rapidly upregulated in the presence of proinflammatory cytokines. E-selectin ligands are present on monocytes, NK cells, memory T cells, neutrophils and eosinophils. Therefore, E-selectin is a key element for recruitment of leukocytes to sites of inflammation. *In vitro*, the major ligand was identified as the Lex terasaccharide, a representative of α2,3-sialylated α1,3-fucosylated glycans. Its in vivo expression pattern however, does not match the presence of E-selectin ligands. Recently, PSGL-1 was identified as an important ligand for E-selectin, but only when it contains the appropriate α2,3-sialic acid and fucose modifications. E-selectin shares some functional redundancy with P-selectin. P-selectin is expressed mainly on platelets and endothelial cells, and is upregulated upon activation with inflammatory stimuli. P-selectin recognizes sialylated and sulphated PSGL-1 and plays an important role in rolling and adhesion of leukocytes to endothelium, as well as the interaction of platelets with monocytes. Once effector cells have reached the site of inflammation, C-type lectins are involved in self-nonself discrimination. For example, the C-type lectins, CD94 and members of the NKG2 family play an important role in NK cell regulation. CD94 and NKG2A form a covalently linked heterodimeric receptor complex, which acts as a killer inhibitory receptor (KIR) (Carretero et al., 1997). If the receptor binds to non-classical HLA-E on the surface of a target cell, an inhibitory signal is generated and ablates activated NK cell cytotoxicity (Braud et al., 1998). C-type lectins, which function as endocytosis receptors include the hepatic asialoglycoprotein receptor, the macrophage mannose receptor, and the intensively studied dendritic cell lectin DEC-205 (Figdor et al., 2002).

P-type lectins are selective for mannose-6-phosphate, which is a critical molecule for protein trafficking to the lysosomal compartment. These particular lectins are important for intracellular trafficking as well as for endocytosis (Dahms and Hancock, 2002).

The family of I-type lectins has become a focus of research interest in recent years. There are several members expressed in the nervous system, such as NCAM and P0. Receptors in the immune system include PECAM, ICAM-1 and CD48. Siglecs (sialic acid-binding Ig-like lectins) are a family of lectins specific

for α2,3- and α2,6-linked sialic acids. These molecules are expressed in tightly regulated patterns on cells of virtually every hematopoietic lineage. Ligands for the different Siglecs were identified by a detailed analysis using synthetic oligosaccharides presented on a streptavidin-alkaline phosphatase support (Blixt et al., 2003). There are eleven known members of the Siglec family and genetic analyses, as well as homology screens, suggest that all human members have been identified (Crocker and Varki, 2001). Siglecs are type II transmembrane proteins and feature an N-terminal V-type lectin domain followed by a maximum of 16 C2-set Ig-like domains. The cytoplasmic carboxy terminus contains tyrosine-based signal transduction motifs. Whereas Siglec-1 (sialoadhesin) does not contain an inhibitory motif, all other Siglecs share one or two tyrosine-based inhibitory motifs (ITIMs). Upon engagement, the tyrosine phosphatase SHP-1, and in some cases SHP-2, is recruited to the cell membrane and activated (Angata et al., 2002). These phosphatases turn off src family tyrosine kinases and thereby block activation signals. In an elegant study, Kelm and colleagues showed that the sialic acid binding sites of CD22 (Siglec-2) on the surface of B cells were engaged in cis interactions with sialic acid moieties (Kelm et al., 2002). However, upon B cell activation binding sites of CD22 opened up and became available to bind ligands in trans on the surface of another cell. Similarly, crosslinking CD33 (Siglec 3) resulted in reduced proliferation of myeloid cells (Vitale et al., 1999). As many members of the recently cloned Siglecs were found on dendritic cells, it will be interesting to see if Siglecs participate in antigen presentation to T cells (Angata et al., 2002).

Another receptor featuring structural similarities with siglecs and reportedly binding sialic acids is CD83. Human CD83 is a 45-kDa glycoprotein and member of the Ig superfamily (Zhou et al., 1992). Its selective expression and up-regulation, together with co-stimulatory molecules such as CD80 and CD86, suggests an important role of CD83 in the immune response (Zhou and Tedder, 1996). CD83 was categorized as a siglec, because the interaction between CD83 and its ligand is dependent on sialylation. Treatment of monocytes and a subset of activated or stressed T cells expressing the CD83 ligand with sialidases abrogated binding (Scholler et al., 2001). Using a plate adhesion assay another group demonstrated that immature as well as mature DCs bind to recombinant CD83-Fc fusion protein (Lechmann et al., 2001). Due to its expression pattern on antigen-presenting cells in T cell-rich areas of the lymph node, CD83 and its potential ligand might also directly contribute to the activation of T cells (Cramer et al., 2000). This hypothesis is corroborated by the analysis of CD83-deficient mice, which have a specific block in $CD4^+$ single positive thymocyte development (Fujimoto et al., 2002). Moreover, CD83-Ig fusion protein was shown to be co-stimulatory when co-immobilized with anti-CD3 for human T cells (Scholler et al., 2002).

Soluble receptors for carbohydrates

As mentioned above, collectins belong to the C-type lectins (Hoppe and Reid, 1994; Holmskov et al., 2003). The human collectins known to date are the mannan-binding lectin (MBL), the surfactant proteins A and D (SP-A, SP-D), CL-L1 (Ohtani et al., 1999) (or collectin liver 1) and CL-P1 (Ohtani et al., 2001). The name collectin reflects their structure, as these proteins contain both a collagenous and a (C-type) lectin carbohydrate-recognition domain (CRD). The collectins are oligomers of trimers of identical polypeptide chains. An exception is SP-A, where the subunits might be made up of heteromers of the two similar polypeptides SP-A1 and SP-A2. The short N-terminal segment of collectins is responsible for oligomerization and the collagenous region of variable length forms triple helices with two other polypeptide chains. A short neck region forming trimeric α-helical coiled-coils links the collagenous region to the C-terminal CRD containing four conserved cysteines. CL-P1 is different from the other collectins in that it contains an N-terminal cytoplasmic domain with an endocytosis motif and a CRD with six conserved cysteines (Ohtani et al., 2001). Collectins have been shown to bind mannose, L-fucose, glucose, maltose, N-acetyl-D-glucosamine and N-acetyl-D-mannosamine, each collectin with a characteristic preference (Lu et al., 2002). The affinity of a CRD for a sole monosaccharide is low (Iobst et al., 1994), but can be increased to a high avidity interaction by multiple binding (Lee, 1992). Microorganisms, but not mammalian cells, exhibit a specific repetitive pattern of terminal sugars and thus, only microorganisms can be specifically recognized by the CRDs of collectins. Collectins have been shown to bind to bacteria, fungi, viruses and protozoa, however, the spectrum of microorganisms and the preference for a given microorganism differs for each collectin (Lu et al., 2002). This binding leads to enhanced phagocytosis or activation of the complement pathway and results in clearance of the pathogen.

Accumulating evidence has suggested that MBL plays an important role in innate immunity (Kilpatrick, 2002a). The basic subunits of MBL are comprised of 24 kDa homotrimeric polypeptides. MBL is thought to exist in oligomers ranging from two to six subunits. Based on electron microscopy, the oligomeric structure shows similar structural features to the complement protein C1q (Lu et al., 1993). MBL is synthesized by the liver and circulates the blood stream. After binding to the respective terminal sugars, MBL indirectly promotes opsonization by initiating the lectin pathway of complement activation. In addition, there is evidence that it can directly act as an opsonin (Kuhlman et al., 1989). Similar to C1q and the proteases C1r and C1s, MBL interacts with MBL-associated serine proteases (MASPs) to initiate complement activation. Three MASPs (MASP-1, -2, -3) and a small MBL-associated

protein (sMAP or MBL-associated protein (Stover et al., 1999; Takahashi et al., 1999)) were identified. Recently, experimental data has suggested that a smaller oligomeric form of MBL complexes with MASP-1 and MAp19 to cleave C3 directly and that a larger oligomeric form of MBL assembles with MASP-2 and MASP-3 to recruit C2/C4 (Dahl et al., 2001). MBL has been shown to bind to a variety of fungi, bacteria, protozoa and viruses (Kilpatrick, 2002a).

Numerous studies have shown the clinical significance of MBL, e. g. children deficient in MBL are particularly susceptible to infections (Kilpatrick, 2002b). In addition, MBL has been reported to be a risk factor for infections for adults (Kakkanaiah et al., 1998). The role of MBL in various diseases has been discussed comprehensively elsewhere (Petersen et al., 2001; Kilpatrick, 2002b).

Human ficolins include L-ficolin (also known as p35), M-ficolin (or ficolin-1) and H-ficolin (known as Hakata antigen) (Lu et al., 2002; Holmskov et al., 2003). The secondary structure of ficolins resembles that of collectins. However, the C-terminal lectin domain consists of a fibrinogen-like CRD directly linked to the collagenous domain (Ichijo et al., 1993; Lu et al., 2002). Ficolins exist in oligomers of subunits of trimeric helices. In contrast to collectins, ficolins bind sugar residues in a Ca^{2+}-independent manner. All ficolins bind to N-acetyl-D-glucosamine. Recently it has been shown that also L-ficolin and H-ficolin, can complex with MASPs and initiate the lectin pathway of complement activation (Matsushita et al., 2000, 2002). L-ficolin and H-ficolin can hence indirectly opsonize microbes, but direct opsonization for ficolins has also been described (Lu et al., 2002). Little is known about the microbial targets of ficolins.

Pentraxins comprise a well-conserved family of pentameric proteins. The C-reactive protein (CRP) is as an acute-phase reactant a key member of the pentraxin family. CRP binds to certain microbes in a Ca^{2+}-dependent manner. CRP activates the classical pathway of the complement system by binding to C1q and can directly act as an opsonin (Du Clos, 2001).

The group of S-type lectins include the immunologically important galectins. To date 14 different galectins have been identified and shown to be multivalent receptors for N-acetyllactosamine. They are soluble proteins, secreted by a non-classical pathway, which bypasses the Golgi compartment. Three of the galectins (galectins 1, 3 and 9), exert very different immunological functions. Galectin-1 is a 14.5 kDa protein and can be found in either a monomeric or homodimeric form. It is a negative regulatory factor in the immune response, reduces lymphocyte proliferation, induces apoptosis in T cells, inhibits secretion of pro-inflammatory cytokines, and regulates cell adhesion (Rabinovich et al., 2002). In contrast, galectin-3, a 29 kDa protein, promotes cell growth, proliferation and induces pro-inflammatory cytokines. The anti-apoptotic role of galectin-3 seems to be an intracellular function and it is interesting that this lectin shares some sequence homology with bcl-2 (Yu et al., 2002). Galectin-9 is less well characterized. It is expressed by eosinophils and seems to be an inhibitory galectin. Galectins mediate their biological activity by clustering proteins on the surface of the target cell with multivalent binding. For galectin-1 it has been shown that CD7 is a key receptor and initiates the apoptotic signal in lymphocytes (Pace et al., 2000; Roberts et al., 2003). Molecules such as CD43, CD2 and CD3 have also been demonstrated to serve as ligands for galectin-1.

In summary, the immune system has developed a network of lectins for regulatory purposes. These lectins can differentiate between various functional states of the target cells. Lectins can initiate as well as modulate immune responses. Activated T and B cells display a lower density of sialic acid residues on their surfaces. The penultimate carbohydrate galactose then becomes available for receptor interaction. While sialic acids are ligands for Siglecs, lactosylamines are ligands for galectins. This balance might play an important role in signalling during immune responses.

Carbohydrates – tools for active immune evasion of tumours?

Changes in glycosylation are early events in the multi-step process of malignant transformation (Hakomori, 2002). Such changes correlate with survival rates of cancer patients (Cajot et al., 1997). These alterations are basic features of malignancies, and may contribute to the loss of contact inhibition, metastasis and immune evasion. For example Demetriou and colleagues have shown that the increased weight of glycoproteins in malignant cells is largely due to increased α1-6-branching of the N-glycans (Demetriou et al., 1995). This reaction is catalyzed by GlcNAc transferase V. This work provided the first piece of evidence that this gene is a classical oncogene. Transfection into formerly normal cells resulted in loss of contact inhibition with subsequent colony formation in soft agar and increase in metastasis formation. This effect could be reversed by addition of swainsonine, an inhibitor of N-glycosylation.

Another interesting observation is the overall increase of sialic acid density in many tumours (Bresalier et al., 1996). The changes in sialic acid content have been investigated in recent years and it has become apparent that this terminal moiety of glycans is of great importance. As discussed earlier modified N-acetylneuraminic acid (sialic acids) serves as ligand for selectins and other adhesion molecules and loss of sialic acids greatly changes adherence properties of the respective cell (Jiang et al., 1992). Therefore, the intercellular communication leading to contact inhibition is disturbed (Demetriou et al., 1995). This might also contribute to the finding that metastatic cells derived from a primary tumour are often characterized by a different pattern of sialylation (Nemoto-Sasaki et al.,

2001). Interestingly, there seems to be a preference for the expression of α2,6-sialic acid, exemplified in human colon carcinomas. Whereas normal colon tissue does not display α2,6-sialic acid moieties, colon carcinoma have increased levels of this particular linkage (Sata et al., 1991). This change is also observed in colon adenoma representing a premalignancy in this kind of tumor (Wang et al., 2001). These changes are not only detectable in adenocarcinomas and epithelial carcinomas (Chen et al., 2002), but also in tumours of the CNS, such as gliomas (Yamamoto et al., 2001).

Perhaps the most prominent proteins overexpressed by tumours are the differentially glycosylated mucins (Bresalier et al., 1996). The role for mucins in tumor invasiveness and immune escape seem to be complex and poorly understood. Although there have been numerous approaches to generate mucin-specific T cells, the therapeutic effect is not satisfactory (Brossart et al., 2001). These T cells infiltrate the tumour, but do not attack it (Agrawal et al., 1998; Mukherjee et al., 2001). It is speculated that altered glycosylation is responsible, in part, for immune escape of tumours. In the latter example, one would postulate that there are mechanisms, by which mucins interfere with antigen presentation or deliver an inhibitory signal to infiltrating T lymphocytes themselves (Vlad et al., 2002). Immune escape by differential glycosylation has been investigated recently and has been shown to correlate with the degree of malignancy of breast cancer (Hakim, 1988). The precise mechanism is still elusive, but there is a possibility of direct interaction with inhibitory receptors on the surface of cytotoxic T lymphocytes and NK cells (Agrawal et al., 1998; Sinclair, 2000). Several paired inhibitory and activating signals derived from the same receptor complex have been proposed (Agrawal et al., 1998; Taylor et al., 2000). It is tempting to speculate that malignant cells may have the capability to engage inhibitory carbohydrate receptors and shift the balance of paired activating and inhibitory signals towards a negative signal. Another way to tip the balance towards a net inhibitory signal is to prevent the activating signal to get through. In this context CD83 could play a significant role. For example, CD83 has been suggested to be a sialic acid-binding receptor and is involved in delivering immune stimulatory signals, mainly to dendritic cells, but potentially to the interacting effector cell as well. CD83 can also be produced by malignant cells, where it is proteolytically cleaved and shed from the surface of the tumour cell to competitively bind CD83 receptors on immune cells. The majority of Hodgkin's cells express CD83 (Sorg et al., 1997). Although there is a soluble form of CD83 in healthy individuals, which is released by activated DC and B lymphocytes, Hodgkin's cells release excessively high amounts of soluble CD83 (Hock et al., 2001). The effect of this process was mimicked, when recombinant soluble CD83 was injected into mice bearing the immunogenic P815 mastocytoma (Scholler et al., 2002). These animals showed a significantly enhanced rate of tumor growth and inhibited development of cytotoxic T cells.

In summary, an array of carbohydrate receptors is relevant to the physiology of an immune response. As our knowledge about the ligands of newly discovered carbohydrate receptors increases, we will better understand the glycan network and lectins utilized by the immune system and perhaps how it is abused by malignant cells to escape from immune effector cells. This may provide useful therapeutic strategies for treatment of a variety of malignancies (Dwek et al., 2002).

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