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Galectin binding proteins in serum and bronchoalveolar lavage -in healthy and pathological conditions

av Cecilia Cederfur



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Akademisk avhandling

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för
avläggande av doktorexamen i medicinsk vetenskap kommer att offentligens försvaras i
Segerfalksalen, Wallenberg Neurocentrum, BMC, Sölvegatan 17, Lund, lördagen den 31 maj
2008, klockan 9.00.

Fakultetsopponent: Professor Peter Pålsson

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Abstract <p>Galectins are carbohydrate binding proteins, implicated in conditions of both inflammation and cancer. Connections between chronic inflammation and cancer are proposed by the increased remodelling and proliferation that occurs, leading to enhanced survival and proliferation of malignant cells. Since galectins have been implicated in mechanisms of both chronic inflammation and cancer, we have investigated natural binding partners of galectins in healthy individuals and then continued with studying states of cancer and chronic inflammation. We identified galectin binding glycoproteins in sera from healthy individuals and found that galectins widely expressed in the body bind serum glycoproteins well, whereas galectins with a more tissue-specific distribution scarcely binds any serum glycoproteins. We then chose the widely expressed but intermediately binding galectin-1 to detect if levels of galectin-1-binding proteins are increased in sera of breast cancer patients. We found that galectin-1 binds approximately double the amount in breast cancer patients compared to healthy individuals. The increase was mainly caused by haptoglobin, probably due to both increased expression and changes of glycosylation.</p> <p>To further investigate the inflammatory connection we identified galectin binding proteins from bronchoalveolar lavage of asthma patients and healthy individuals, additionally we compared the binding of galectin-3 and galectin-8 that are expressed in different sites of the lung. We found when functionally grouping the bound proteins that galectin-3 and -8 binding proteins had different profiles and that bound proteins of healthy and asthma patients differed.</p>		
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Cecilia Cederfur

**Galectin binding proteins in serum and
bronchoalveolar lavage**

-in healthy and pathological conditions

Department of Laboratory Medicine

Section of Microbiology, Immunology and Glycobiology

Lund University, 2008



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Medicinska fakulteten

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What is a scientist after all?

It is a curious man looking through a keyhole, the keyhole of nature,

trying to know what's going on.

Jacques Yves Costeau

ABSTRACT

Galectins are carbohydrate binding proteins, implicated in conditions of both inflammation and cancer. Connections between chronic inflammation and cancer are proposed by the increased remodelling and proliferation that occurs, leading to enhanced survival and proliferation of malignant cells. Since galectins have been implicated in mechanisms of both chronic inflammation and cancer, we have investigated natural binding partners of galectins in healthy individuals and then continued with studying states of cancer and chronic inflammation. We identified galectin binding glycoproteins in sera from healthy individuals and found that galectins widely expressed in the body bind serum glycoproteins well, whereas galectins with a more tissue-specific distribution scarcely binds any serum glycoproteins. We then chose the widely expressed but intermediately binding galectin-1 to detect if levels of galectin-1-binding proteins are increased in sera of breast cancer patients. We found that galectin-1 binds approximately double the amount in breast cancer patients compared to healthy individuals. The increase was mainly caused by haptoglobin, probably due to both increased expression and changes of glycosylation.

To further investigate the inflammatory connection we identified galectin binding proteins from bronchoalveolar lavage of asthma patients and healthy individuals, additionally we compared the binding of galectin-3 and galectin-8 that are expressed in different sites of the lung. We found when functionally grouping the bound proteins that galectin-3 and -8 binding proteins had different profiles and that bound proteins of healthy and asthma patients differed.

LIST OF PAPERS¹

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

- I** **Cecilia Cederfur**, Emma Salomonsson, Jonas Nilsson, Adnan Halim, Christopher Öberg, Göran Larson, Ulf Nilsson, Hakon Leffler. Different affinity of galectins for human serum glycoproteins: galectin-3 binds many protease inhibitors and acute phase proteins, *Glycobiology*, 2008 May;18(5):384-94.
- II** **Cecilia Cederfur**, Mårten Fernö, Håkan Olsson and Hakon Leffler. Large increase of galectin-1-binding serum glycoproteins in breast cancer patients. Manuscript.
- III** **Cecilia Cederfur**, Johan Malmström, Kristian Nihlberg, Leif Bjermer, Gunilla Westergren-Thorsson and Hakon Leffler. Identification of galectin-3 and -8 ligands in bronchoalveolar lavage of mild asthmatics and healthy subjects. Manuscript.

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NON-STANDARD ABBREVIATIONS

CRD	carbohydrate recognition domain
APP	acute phase protein
AGP	alpha1-acid glycoprotein
ER	endoplasmic reticulum
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
IL	interleukin
TNF	tumor necrosis factor
TGF	transformed growth factor
MS	mass spectrometry

INTRODUCTION

Reading the “glyco-code”

Cells in the body use various “labels” (tags) to communicate with other cells and also to direct their own building blocks within the cells. One type of such “label” is made of complex carbohydrates, attached to proteins (in glycoproteins) or lipids (in glycolipids) (Fig. 1). Complex carbohydrates are made of monosaccharide strings of different types and linked to each other in different ways, and can thus provide a rich library of specific labels for the cells to use. There are many well known examples where this occurs. For example, carbohydrates determine how certain proteins find their correct destination within a cell, how white blood cells find their correct way around the body, and how virus (such as influenza) and bacteria bind to host-cells, and they also mark part of our individuality as blood group antigens (1-4). To read the carbohydrate “labels”, the cell uses carbohydrate binding proteins, so called lectins that bind specific motifs within the carbohydrate strings. One class of these are the galectins (5, 6) which are the topic of this thesis. The galectins recognize the monosaccharide galactose (white ring in Fig. 1), but their binding also depends on the adjacent sugar residues.

It is still unclear how galectins read natural carbohydrate “labels”, what the biological function of this is, and how this can be used in medical practice. In this thesis these questions are addressed by the study of which serum glycoproteins are bound by different galectins. Focusing on these questions, serum provides a complex mixture of well characterized natural glycoproteins which the ability of galectins to read carbohydrate “labels” can be studied against. Serum also provides a source of functionally relevant galectin ligands. Detection of galectin ligands in serum may provide a useful tool in diagnosis of

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disease which is focused on in this thesis by the study of galectin ligands in sera of breast cancer patients. The study is extended to another bio-fluid: airway secretions obtained as bronchoalveolar lavage (BAL) where the aim of the thesis is further focused on the biological mechanism of galectins in the inflammatory pathology.

As background, the galectins are first introduced, the main type of glycoproteins found in humans, and how they interact. An overview is given of the constituents of human serum and some examples of bound glycoproteins. Since the studies were compared to both conditions of cancer and chronic inflammation a brief overview will be given over these large areas together with implicated roles of galectins.

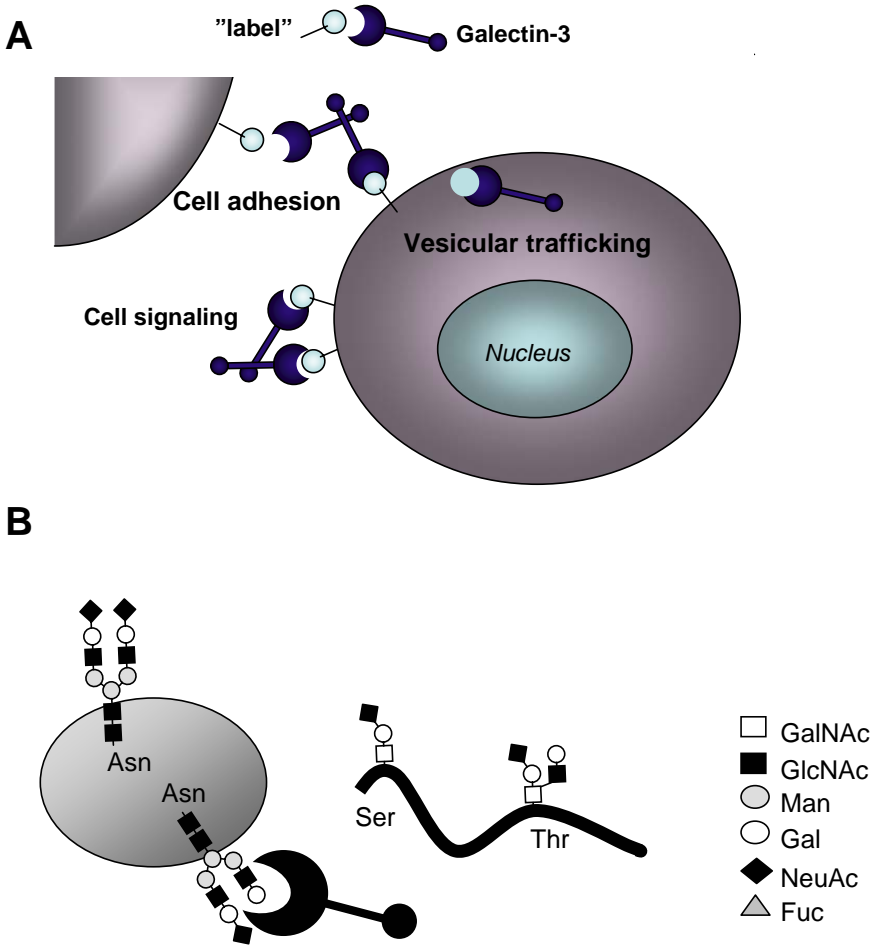


Fig. 1. Reading the “glyco-code”

A) Examples of cellular functions that involve binding of galectin-3 to a carbohydrate “label”.

B) More details at the glycoprotein level, with schematic glycans and recognition by galectin-3. Different monosaccharide residues are shown schematically according to the recommendation of Consortium for Functional Glycomics.

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Introduction to galectins

The galectins are small soluble proteins, defined by a carbohydrate recognition domain (CRD) and a conserved sequence motif (6). There are about 15 galectins, some, such as galectin-1, -3, -8 and -9 are widely distributed in many cell types, whereas others like galectin-2, -4 and -7, have a much more restricted distribution (7-11) (Fig. 2).

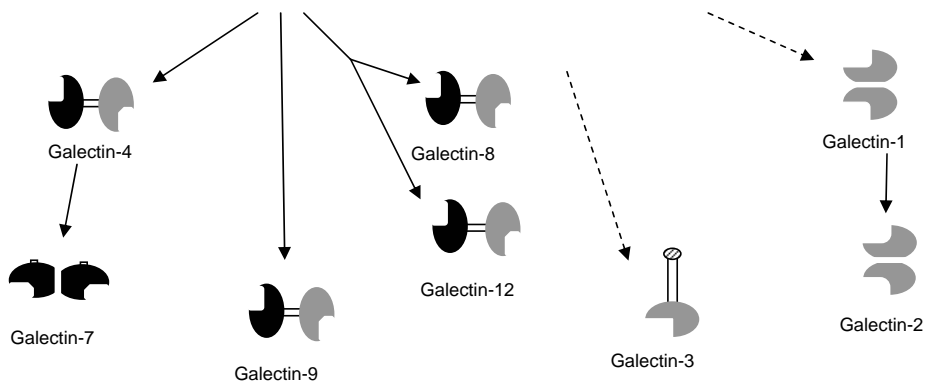


Fig 2. The major conserved mammalian galectins, and their phylogeny in vertebrates. All galectins have CRDs of either of two types (here shown as black or grey) and can be either mono-, or bi CRD galectins. The two types of CRDs are defined by intron-exon boundaries and supported sequence relationship.

Physiological roles of galectins

On the cellular level galectins are involved in mechanisms both inside the cell and extracellularly. Galectins have been proposed to play important roles in inflammation, immunity and cancer based on whole animal experiments and by

BACKGROUND

their effects in tissue culture (12-17). Intracellularly, galectins are found in the cytosol and nucleus where they are involved in targeting exocytosis, cell activation and differentiation (18-20). Galectins are also secreted by different cells via the non-classical pathway and can attach to sugar structures on e.g. glycoproteins on the cell surface (21) on the secreting cell itself or other cells in the vicinity. There they crosslink glycoproteins and perhaps glycolipids and form lattices on the cell surface that are believed to activate receptors and mediate signalling to the inside of the cell (7, 22-24) (Fig. 3). By binding preferably N-glycosylated proteins that may reside in solution as serum proteins (25, 26) or on the cell surface, galectins modulate cell adhesion (27, 28) and can extend the time glycoproteins as the EGF receptor stay on the cell surface (29, 30). Functions are being specific in some cases, but also general between galectins in others (14, 15). It has become evident that protein-carbohydrate interactions are important in the communication between cells and with the extracellular matrix, as well as communication within the cell (2). There is, however only little knowledge on how natural ligand binding and effects are related to galectin fine specificity (13, 31-37).

BACKGROUND

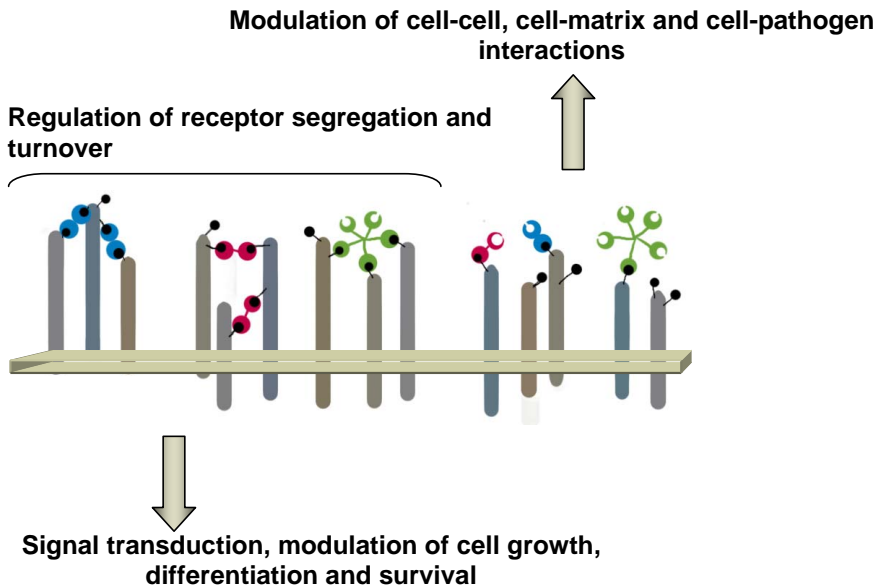


Fig. 3. Functional relevance of galectin-glycoprotein lattices. Representation of different monomeric and oligomeric members of the galectin family in galectin-glycoprotein lattices, showing galectin-glycoprotein interactions at the cell surface and possible biological effects. Figure adapted from (38).

Galectin expression

Some of the galectins are widely expressed in the body whereas others are only expressed in distinct compartments (Table 1). Within the immune system, galectins are found in activated macrophages, activated B cells, dendritic cells and activated T cells (20, 39-42). Endothelial cells express several galectins (43).

Galectin-1 is expressed in human cultured endothelial cells, in aorta, umbilical vein and pulmonary artery, in vivo it was found in activated lymphoid tissue but not in resting lymph nodes (44). Galectin-3 expression has been observed in epithelial cells, macrophages, fibroblasts and activated T-cells (45). In endothelium it was found in e.g. dermal microvasculature and in cultured

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cells it is expressed by endothelial cells originating from human umbilical veins (HUVECs). Galectin-9, was originally identified as an eosinophil specific chemoattractant (46) and is also expressed in cultured cells as HUVECs (8, 9, 44). Galectin-8 is expressed in lung (47) both in basal cells, ciliated bronchial cells, chondrocytes, serous cells of the bronchial glands, smooth muscle cells and endothelial cells (48).

Table 1. Localisation and function of the different galectins. The table gives examples of the many functions and the wide distribution of galectins (39, 49-51).

Galectin	Localization	Functions and other characteristics
Gal-1	Abundant in most organs, e.g. lymph nodes, spleen, thymus, placenta, macrophages, B-cells, T-cells.	Non covalent homodimer * Induces apoptosis of activated T-cells and immature thymocytes. * Induces a Th2 immune response. * Modulates cell-cell and cell-matrix interactions. * Inhibits acute inflammation.
Gal-2	Stomach epithelial cells	Non covalent homodimer * Expressed at minor levels in epithelial cells.
Gal-3	Macrophages, epithelial cells, fibroblasts and activated T-cells.	Chimera type galectin (non-lectin domain coupled to the CRD) * Antiapoptotic and proinflammatory functions. * Modulates cell adhesion and migration. * Induces chemotaxis of monocytes. * Potentiates pro-inflammatory cytokine secretion. * Inhibits nitric oxide-induced apoptosis and anoikis (loss of cell attachment).
Gal-4	Gastrointestinal tract	Composed of two distinct CRDs linked by a polypeptide chain
Gal-5	Erythrocytes	Prototype galectin, monomer
Gal-6	Gastrointestinal tract	Composed of two distinct CRDs linked by a polypeptide chain * Similar to galectin-4

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Gal-7	Skin	Prototype galectin, monomer * Used as a marker for stratified epithelium.
Gal-8	Liver, kidney, cardiac muscle, prostate and brain.	Composed of two distinct CRDs linked by a polypeptide chain * Modulates integrin interactions with the extracellular matrix.
Gal-9	Thymus, T-cells and kidney.	Composed of two distinct CRDs linked by a polypeptide chain * Induces eosinophil chemotaxis. * Induces apoptosis of murine thymocytes.
Gal-10	Eosinophils and basophils	Prototype galectin, monomer * Mainly expressed by eosinophils * Forming the Charcot-leyden crystals
Gal-11	Lens	Non covalent homodimers * Also called GRIFIN * May represent a new lens crystalline. * Lacks affinity for β -galactoside sugars.
Gal-12	Adipocytes	Composed of two distinct CRDs linked by a polypeptide chain * Induces apoptosis and cell cycle arrest.
Gal-13	Human placenta	Similar to prototype galectins * Also known as PP-13.
Gal-14	Eosinophils	Prototype galectin
Gal-15	Endometrial epithelium	Contains a conserved carbohydrate recognition domain and a separate putative integrin binding domain.

Some members of the galectin family have also received individual names according to their functions, localization or biochemical properties, including galectin-1 (L-14, bovine heart lectin or galaptin), galectin-3 (Mac-2, L-29, CBP-35 or α BP for 'IgE-binding protein'), galectin-9 (ecalectin), galectin-10 (Charcot-Leyden crystal eosinophil protein), galectin-11 (GRIFIN for galectin-related interfiber protein) and galectin-13 (PP-13)

Galectin-carbohydrate interactions

The galectin carbohydrate binding specificity has been characterized extensively against panels of small saccharides, and the structural basis for the interaction determined in some cases by X-ray crystallography and NMR-spectroscopy (6, 52, 53).

The galectin CRD has about 130 amino acids folded as a slightly bent β -sandwich forming a groove on the concave side (Fig. 4). This groove forms the galectin carbohydrate recognition site with 5 subsites (A-E) (10). Subsite C is the defining conserved binding site of the galectin CRDs, and gives them their shared specificity for β -galactose residues. The other subsites determine the variable fine specificity for larger saccharides, where additional saccharide moieties are attached to the galactose. Thus, addition at position 1 of 4Glc (as in lactose), 4GlcNAc (as in LacNAc), 3GlcNAc or 3GalNAc are bound in site D, and enhances binding to different degree for different galectins. Addition to position 4 and 6 of the Gal block galectin binding, as these positions point into the protein surface (Fig. 3). Additions to position 3, however, point into site B, and are tolerated or enhance binding to varying degrees. Of special interest here is NeuAc α 2-3, which, for example, is tolerated by galectin-3, but strongly preferred by the galectin-8 N terminal CRD. Additions to position 2 e.g. Fuc α 1-2, are also tolerated, or may enhance binding. Saccharide moieties potentially bound by galectins based on the criteria described above, are found in glycoproteins, and in glycolipids, but the extent to which galectins really bind them is less well known. Additional interactions e.g. in the loosely defined site E, may enhance or prevent binding to a saccharide when present in its natural context as part of a glycoprotein or glycolipid.

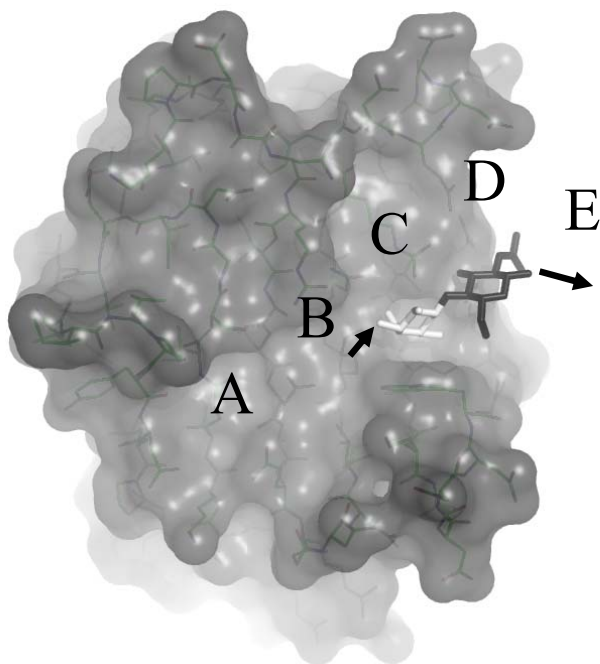


Fig. 4. Molecular model of the galectin-3 carbohydrate recognition domain (CRD) with bound LacNAc. The protein is shown as a solvent accessible surface, rendered semitransparent to show inside residues in wire format. The bound sugar is shown in stick model with the Gal residue white and the GlcNAc black. The localization of binding subsites are indicated by A-E and the linkage position of permissible extensions in site B and further extensions into site E are indicated by arrows.

The protein glycosylation machinery

Most secreted proteins, proteins exposed at the cell surface, and proteins exposed to the luminal side of intracellular vesicles reach their destination via the “classical” ER-Golgi pathway. While being synthesized on ER-bound ribosomes, the proteins enter the ER-lumen, in full for secreted proteins, or partially for transmembrane proteins. From there the peptide chain is moved through the ER, Golgi and subsequent trafficking vesicles to reach their

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destination ([54-57](#)). On, the way, most are modified with sugar chains in reactions catalyzed by glycosyltransferases while they pass through the ER-Golgi compartments. There are different types of glycosylations, as exemplified in Fig. 1B and below). Since N-glycans appear to provide the major, if not only, cell surface binding site for galectins ([34](#)), and are also the major glycan type found on serum glycoproteins, they are described in some extra detail below.

N-linked glycans

N-glycan addition occurs on asparagines in the sequence context Asn-X-Ser/Thr, via a complex synthetic machinery illustrated in Fig. 5. The first part of this machinery (top panel of Fig. 5) is conserved in all eukaryotic organisms ([57](#)), and is functionally involved in proofreading protein folding and early transport. The synthesis of a 14-sugar precursor lipid-linked oligosaccharide is begun in the cytosol and completed after flipping into the ER-lumen. The glycan is then transferred to a newly synthesized peptide chain entering the ER-lumen, if it has an available asparagine in the correct sequence context. Three glucose units and up to six mannoses are then removed from many glycans before the proteins enters the cis-Golgi. The last glucose, which can be removed and re-added, is recognized by the ER-lectin-chaperones calnexin and calreticulin as a signal of improper/incomplete folding. The mannoses are recognized by the membrane protein lectin ERGIC-53, which is involved in transport of proteins between ER and cis-Golgi. The CRD of ERGIC-53 has the same β -sandwich fold type as galectins, but does not recognize galactose ([58, 59](#)).

Galactose residues, and thereby potential galectin binding sites, are added in the next part of the protein glycosylation pathway (middle panel of Fig. 4), which is conserved in vertebrates and some other multicellular organisms, but not unicellular ones. GlcNAc residues are added to the core of the inner most three mannose residues to initiate branches (referred to as antennae), and usually galactose is quickly added to the GlcNAc. The enzymes that initiate the

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antennae are GlcNAc-transferases (I-VI) and are encoded by the *Mgat1-6* genes. They act in a precise order and are highly regulated, with activities varying due to cellular conditions. For example, *Mgat1* must act before any of the others can act; *Mgat5* is responsible for addition of a third antenna that has a strong correlation with cancer (29, 30). The result is N-glycans with 2-4 (or even 5) antennae, collectively named complex structures. A few “hybrid glycans” get only one antenna and retain two additional mannoses on the other side, and some glycans also remain as “high mannose structures” with no addition of GlcNAc residues.

Galactose is usually added to the antenna GlcNAc in β 1-4 linkage to produce a LacNAc, but 1-3 linkage also occurs; both provide a galectin binding site (indicated by vertical bar in Fig. 5). The complex N-glycan can now be further modified, mainly in the later parts of the Golgi, by addition of sialic acid (NeuAc), fucose, further extension of the antennae (by additional LacNAc residues added to the first), and other modifications. This may or may not block galectin binding sites, or create new ones, as exemplified in Fig. 5, bottom panel.

Mature glycoproteins, e.g. as found in serum, may contain one or more N-glycans selected from the type of structure shown in the two bottom panels of Fig.4. Thus the more fully processed complex structures tend to predominate, whereas earlier high mannose and hybrid structure are rarer. The structures from the ER (top panel in Fig. 5) are usually not found on mature glycoproteins, but do occur. Multiple N-glycosylation sites on the same protein may contain different glycan structures, and the glycans found at one particular site also varies in a protein sample. Even if fully processed forms may predominate, partially processed forms also occur, lacking one or more terminal modifications, or having, by regulation, different number of antennae. This microheterogeneity is experimentally observed by the fact that a particular glycoprotein may appear as an indistinct band on SDS-PAGE, produce many

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spots on isoelectric focusing and multiple masses on mass spectrometry. It is functionally regulated in many important contexts such as inflammation and cancer (29, 60, 61), and, as discussed below determines the degree of binding to galectins.

Varied expression levels of glycosyltransferases can affect the repertoire of N-glycan structures produced in the specific cell type. Some glycosyltransferase and glycosidase genes contain promoter regions bearing transcription-factor-binding elements that function in growth regulatory and oncogene transformation pathways. Substrate levels have also been reported to influence the branching of N-glycans. The Golgi pathway is sensitive for hexoseamine levels which affects the production of tri and tetra antennary N-glycans. Glycoproteins can be joined in a lattice prolonging their time on the cell surface before endocytosement and recycling (7, 29, 62). Galectin-3 is involved in attachment of the proteins in the lattice and binds N-glycans with α 2,3-sialylated or free galactoses. More N-glycans on a protein and more branches on N-glycans increase the probability for galectin-3 binding. This leads to an increased retainment of N-glycosylated proteins as the EGFR on the cell surface. In line with this, proteins with several N-glycan sites have been shown to be less sensitive than proteins with few sites that require higher hexosamine levels to prolong the time the protein stays on the cell surface (29).

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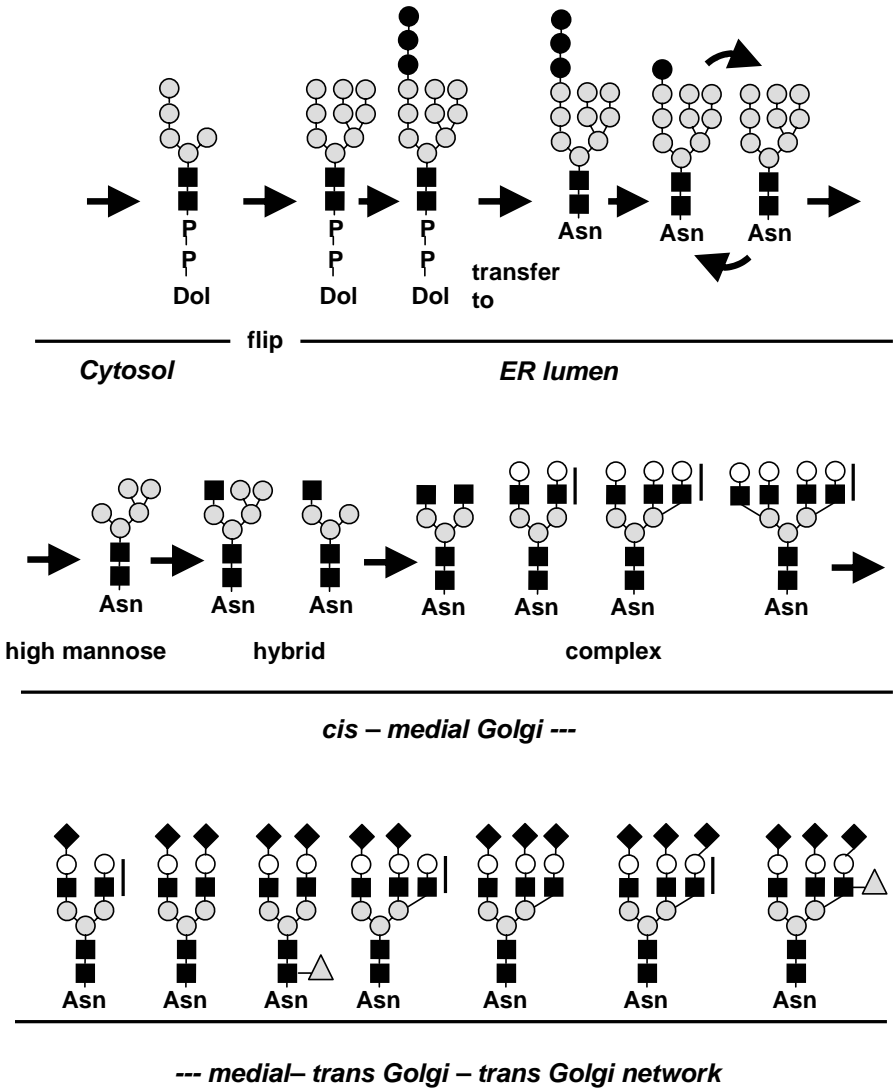


Fig 5. The N-glycosylation pathway. Saccharides are shown in the same symbols as in (Fig 1B), based on (57). Potential galectin binding sites based on saccharides are marked by a vertical line. For the multiantennary structures in the middle panel, only one binding site is marked even if there are more. In the bottom panel, sialic acids (diamonds) are linked to the 6 position of Gal, except the most right one in the two last structures, which are linked to the 3 position, as indicated by their oblique connection.

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O-linked glycans

The most common O-linked glycosylation on cell surface and extracellular glycoproteins starts after the addition of GalNAc to serine or threonine of a protein in the Golgi. Sugars are then added to form disaccharides and many can be extended by sequential addition of galactose, GlcNAc, fucose and sialic acid to generate linear or multi-branched chains (1, 56) (see examples in Fig. 1B). In contrast to N-glycosylation, a consensus sequence for GalNAc addition to polypeptides has not been found.

A galactose can, for example, be added to position 3 of the first GalNAc, to create a Core 1 structure (also named T-antigen). If a GlcNAc also is added to the 6 position of the GalNAc, the structure is called Core 2, which can be extended on the GlcNAc by repeating Gal and GlcNAc residues. Similarly but with addition of GlcNAcs Core 3 and 4 structures are created. Less common structures are Core 5-7. If the Gal of Core 1 is extended by NeuAc α 2-3, no further modifications can occur, and the structure is also referred to as sialyl-T-antigen (T) antigen.

Since, there are many β -galactose residues on O-linked glycan, there are many potential galectin binding sites. However, like in N-linked glycans, these sites can be blocked or enhanced depending of their context and further modifications. The T-antigen (Gal β 1-3GalNAc) and its sialylated version, are excellent ligands for galectin-8N, but poor ligands for galectin-3, even if the latter has attracted a lot of attention (63). Extended LacNAc containing chains in Core-2 glycans on the other hand, may be good ligands for galectins-1 and -3.

O-glycans can often occur in clusters with many glycans attached to Ser or Thr residues near each others. These clusters are especially large in mucin-type molecules that occur at the cell surface and as secreted glycoproteins on mucosal surfaces. Other proteins have smaller clusters of O-glycan often in regions connecting other domains of the protein, for example as found in IgA. Finally some proteins contain relatively few scattered O-glycans. In some cells, for

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example certain circulating blood cells, O-glycans can be as abundant as N-glycans, but on serum glycoproteins, O-glycans are much scarcer. In mucins, the many O-glycans contribute to general physical properties such as retention of water and ions, and protective gel formation. O-glycans can also act as specific “tags” being recognized by lectins. For example, the counter receptor of selectins involved in the extravasation of leukocytes, often carries O-glycans with the specifically recognized sialyl-Le^x structure (4).

Ser and Thr residues in proteins can also carry O-linked saccharides, such as GlcNAc in the cytosol, Xyl that initiate glycosaminoglycan addition, and Fuc and Man that initiate more unusual short chains involved in specific signalling (1, 3, 57). However, these are less relevant for galectin binding, as far as is known, and do not occur on serum glycoproteins.

Glycolipids

Glycosphingolipids are characterized by an O-linkage between glucose (sometimes galactose) and a ceramide. In larger glycosphingolipids, a Gal β 1-4 is first added to the Glc forming lactose. This is the only glycan type that contains lactose except for the free saccharides in milk. The lactose may be extended in various ways by adding first GlcNAc, GalNAc or another Gal and then further. This produces different glycosphingolipid series (56, 57), and some clearly are able to bind galectins, e.g. gangliosides. Glycosphingolipids occur in lipid rafts and their glycans bind to each other or e.g. integrins through which they can affect signalling. Galectin-4 has been inferred as a possible cross-linker and stabilizer in lipid rafts (18, 64, 65). The glycosphospholipid anchors, another glycolipid type, contain mannose and glucosamine that are assembled in the ER on a phosphatidylinositol backbone. The glycolipid is then transferred to proteins and where they function as anchors into the membrane, and affect membrane diffusion, intracellular protein sorting and signalling. They are not expected to interact with galectins, as they do not contain galactose.

Galectins and glycoproteins

If a galectin binding site is available, the next question is whether binding will be strong enough to be relevant, and what its functional effect will be. For long it was thought that protein carbohydrate interactions are too weak to be biologically effective, and that multivalent interactions would be necessary (66). However, some monovalent galectin-ligand interactions clearly are strong enough (K_d in nM range) (67), whereas others require divalency (or higher), for example, the likely case for galectin-1 (33). For binding to a particular glycoprotein, a monovalent interaction would require one available binding site, whereas divalent interaction would require two. This may be the reason why galectin-1 binds a smaller subset of serum glycoproteins compared to galectin-3 as found in Paper I.

If we now assume galectin binding has occurred, the following question is what is the functional effect? Here di- or multivalency of the galectin appears to be decisive. It may be to induce signalling by cross-linking a particular ligand, by similar mechanisms as in other cases of receptor cross linking. It may also be to induce larger cross linked lattices of one or more glycoproteins at a cell surface (7, 35). This in turn may result in either signalling as described above or segregation of the cross-linked glycoproteins from other receptors, with consequent effects (Fig. 3). Finally, galectin cross-linking may confer binding of one glycoprotein to another, e.g. a soluble serum glycoprotein to a cell membrane glycoprotein.

Galectins have been reported to interact with a wide range of extracellular and cellular glycoproteins, and many functions suggested as exemplified throughout this text. However, it has remained largely unclear how each particular glycoprotein binds a galectin, and how this is related to the ensuing biological effect. Some examples are given above under galectin function, and below in conjunction with galectins in inflammation and cancer.

Serum proteins

In paper I we studied what kinds of serum glycoproteins galectins bind. Below follows first a definition of serum and an overview of the proteins it contains. Then follows examples of when serum protein levels are shifting with the focus on glycosylated proteins and some specific examples of galectin ligands.

Overview of protein composition of serum

Plasma can be defined as the liquid phase of blood including proteins but excluding cells (68, 69). Serum is the protein solution left after plasma is allowed to clot and thus lacks fibrinogen and prothrombin. The term plasma or serum proteins, generally refers to proteins that fulfil their function in the circulation. However serum also contains a great deal of other proteins: intracellular proteins that leak out into the interstitial fluid and passes on into the blood stream e.g. peptide hormones or as a result of tissue damage e.g. myoglobin, after a heart attack. Thus serum contains known proteins over a very wide concentration range (from mg/ml to pg/ml), which can be divided into functional groups as follows:

Proteins produced by solid tissues, with their function in serum. The classical serum proteins are mainly produced by the liver and intestines and have a molecular mass larger than the kidney filtration cutoff (~45 kDa) and thus a long serum lifetime. The predominant serum protein is albumin (50 mg/ml), and others occur mainly in the range of concentration from the low mg/ml (e.g. transferrin, immunoglobulins, haptoglobin, protease inhibitors, complement factor C3) to the low µg/ml (C-reactive protein, complement factor C2). The serum proteins fulfil a range of functions for example maintaining the colloid osmotic pressure (albumin), transporting low-molecular compounds that are insoluble in water, toxic (e.g. bilirubin and fatty acids) or sparsely occurring (e.g. iron). Some proteins with low molecular mass are bound in complexes to plasma proteins to avoid elimination through glomerular filtration.

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If excluding albumin, the main part of the serum proteins are involved in the defence mechanism of the body, for example protease inhibitors, immunoglobulins and the complement system. In many cases the proteins are also synthesized by other sources than hepatocytes and intestinal cells, for example by B-lymphocytes (immunoglobulins), macrophages (alpha-1-antitrypsin) and endothelial cells, however the hepatocytes dominate the synthesis quantitatively except for immunoglobulins.

Proteins leaking into serum from damaged tissue. These occur at much lower concentrations than the classical serum proteins (~ 0.1 ng/ml – 0.1 μ g/ml). In this category, proteins are included that normally have their function inside the cell but are released into plasma compartment after cell death or damage. Examples are cardiac troponins or myoglobin used for diagnosing myocardial infarction.

Abnormal secretions. This group contains proteins that are released from tumors and other diseased tissues and can be non serum associated proteins released into the circulation by cancer cells. Example of a biomarker in use is the carcino embryonic antigen (CEA) used to detect pancreatic cancer (70).

Long distance and local receptor ligands. They occur at sub ng/ml concentrations. Peptide and protein hormones as insulin and erythropoietin are examples of long distance receptor ligands. The molecular sizes in the group are varying, indicating that time in circulation differs and hence time of activity. Cytokines and other short range mediators of cellular responses are examples of local receptor ligands. They usually have a short lifetime in serum and seem to mostly mediate local effects since they are diluted to ineffective levels in sera and are harmful at high levels in the circulation.

Foreign proteins. These include proteins produced and released into circulation by parasites or other infectious organisms.

Serum proteins of all abundance levels have been shown to be useful as diagnostic tools. Hypothetically all tissue proteins found in serum would be

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candidate damage markers. However that demands knowledge of the normal background levels of tissue destruction and remodelling in a healthy individual.

Glycans of serum proteins. Many serum proteins are glycoproteins, and carry N-glycans, whereas O-glycans are less common. The N-glycans of serum have been characterized extensively, and recently a quantitative profile has been obtained with the help of improved chemical methods for glycan release and fractionation combined with mass spectrometry (71). Table II shows such a profile, where the glycans has been grouped based on their structural features and potential ability to bind galectins. As can be seen, the major N-glycan of all serum glycoprotein is a bi-antennary complex structure with each galactose capped with 6 linked NeuAc. Therefore, this glycan cannot bind galectins. However, as is evident from Table II, serum contains many other glycans that have available galectin binding sites due to incomplete sialylation, additional antennae, and/or sialylation at position 3 of Gal instead of at position 6. The most prominent O-glycosylated prtein of human serum is IgA, which carries mainly sialyl-T-antigen (72), providing a preferred binding site for galectin-8N (67).

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Table II. N-glycans in human serum, concentrations^a, and availability to galectin binding.

No.	Structure	Non-sialylated galactose	Conc. (μM)	In IgG (μM)	Estimated available to galectin (μM)
Two or more non-sialylated galactose			43	15	28
28	NeuAc ₁ (GalGlcNAc) ₄ Man ₃ GlcNAc ₂	3	9	0	9
21	(GalGlcNAc) ₃ GlcNAc Man ₃ GlcNAc ₂	3	5	0	5
31	NeuAc ₂ (GalGlcNAc) ₄ Man ₃ GlcNAc ₂	2	2	0	2
24	NeuAc (GalGlcNAc) ₃ Man ₃ GlcNAc ₂	2	5	0	5
7	(GalGlcNAc) ₂ Man ₃ GlcNAc ₂	2	6	0	6
11	(GalGlcNAc) ₂ Man ₃ GlcNAc ₂ Fuc	2	14	14	0
13	(GalGlcNAc) ₂ GlcNAc Man ₃ GlcNAc ₂	2	1	0	1
18	(GalGlcNAc) ₂ GlcNAc Man ₃ GlcNAc ₂ Fuc	2	1	1	0
One non-sialylated galactose			162	50	113
33	NeuAc ₃ (GalGlcNAc) ₄ Man ₃ GlcNAc ₂	1	2	0	2
27	NeuAc ₂ (GalGlcNAc) ₃ Man ₃ GlcNAc ₂	1	14	0	14
29	NeuAc ₂ (GalGlcNAc) ₃ Man ₃ GlcNAc ₂ Fuc	1	4	0	4
16	NeuAc (GalGlcNAc) ₂ Man ₃ GlcNAc ₂	1	74	0	74
19	NeuAc (GalGlcNAc) ₂ Man ₃ GlcNAc ₂ Fuc	1	22	11	11
20	NeuAc (GalGlcNAc) ₂ GlcNAc Man ₃ GlcNAc ₂	1	3	0	3
23	NeuAc (GalGlcNAc) ₂ GlcNAc Man ₃ GlcNAc ₂ Fuc	1	7	4	4
4	GalGlcNAc GlcNAc Man ₃ GlcNAc ₂	1	1	0	1
6	GalGlcNAc GlcNAc Man ₃ GlcNAc ₂ Fuc	1	29	29	0
12	GalGlcNAc GlcNAc ₂ Man ₃ GlcNAc ₂ Fuc	1	6	6	0
Fully sialylated tri- and tetranatennary			53	0	27
34	NeuAc ₄ (GalGlcNAc) ₄ Man ₃ GlcNAc ₂	0	1	0	1
30	NeuAc ₃ (GalGlcNAc) ₃ Man ₃ GlcNAc ₂	0	39	0	20
32	NeuAc ₃ (GalGlcNAc) ₃ Man ₃ GlcNAc ₂ Fuc	0	13	0	6

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Table II continued. N-glycans in human serum, concentrations^a, and availability to galectin binding.

No.	Structure	Non-sialylated galactose	Conc. (μM)	In IgG (μM)	Estimated available to galectin (μM)
	Other structures		486	24	0
22	NeuAc ₂ (GalGlcNAc) ₂ Man ₃ GlcNAc ₂	0	416	0	0
25	NeuAc ₂ (GalGlcNAc) ₂ Man ₃ GlcNAc ₂ Fuc	0	22	0	0
26	NeuAc ₂ (GalGlcNAc) ₂ GlcNAc Man ₃ GlcNAc ₂ Fuc	0	7	0	0
5	NeuAc GalGlcNAc Man ₃ GlcNAc ₂	0	2	0	0
9	NeuAc GalGlcNAc Man ₃ GlcNAc ₂ Man	0	2	0	0
10	NeuAc GalGlcNAc GlcNAc Man ₃ GlcNAc ₂	0	2	0	0
15	NeuAc GalGlcNAc GlcNAc Man ₃ GlcNAc ₂ Fuc	0	3	0	0
17	NeuAc GalGlcNAc GlcNAc ₂ Man ₃ GlcNAc ₂	0	2	0	0
1	Man ₂ Man ₃ GlcNAc ₂	0	2	0	0
2	Man ₃ Man ₃ GlcNAc ₂	0	4	0	0
3	GlcNAc ₂ Man ₃ GlcNAc ₂ Fuc	0	21	21	0
8	GlcNAc ₃ Man ₃ GlcNAc ₂ Fuc	0	3	3	0
Total			751	89	167

a) Table from paper I. The glycan numbers and structures are shown as interpreted from (71) based on known serum N-glycan structures.

Serum proteins in inflammation and the acute phase response

The acute-phase response is a summary name for a complex series of reactions initiated in response to infection, physical trauma, or malignancy, with the purpose to prevent ongoing tissue damage, isolate and destroy the infective organism and activate the repair processes. It includes changes in the plasma concentrations of certain proteins, thus named acute phase proteins (APPs), as part of a large number of systemic manifestations that replace normal homeostasis during inflammatory states (73). Most APPs are glycoproteins. APP changes are not limited to acute inflammation but persist during chronic inflammation. They are largely a result of alterations in synthesis by hepatocytes in response to circulating cytokines. Although other cells as macrophages, fibroblasts, epithelial cells and adipocytes also can synthesise APPs, these cells are not likely to contribute significantly to plasma concentrations. In humans, infection, trauma, inflammatory disorders and severe malignancies are causing raised levels of APPs. APPs are individually regulated and different patterns are seen in different diseases. A number of inflammation-associated cytokines are important in regulation of the APP response including TNF- α , interleukin-6 (IL-6), IL-1, IL-8 and TGF- β . These cytokines are produced by a number of cells including neutrophils, lymphocytes, fibroblasts, endothelial and epithelial cells although monocytes and macrophages at the inflammatory site constitute the major source. Cytokines also mediate glycosylation changes independently of their effects on APP production which differ between acute and chronic inflammation.

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Examples of galectin binding serum glycoproteins

-functions of the individual proteins and alterations of glycosylation

Alpha-1-acid glycoprotein (AGP) is an acute phase protein that is primarily synthesised by hepatocytes. Various inflammatory cytokines and steroid hormones influence the expression and increased levels have been observed in patients of various inflammatory conditions. AGP has five N-glycan residues that are glycosylated differently during acute, chronic inflammation (74) and cancer (75). AGP is involved in modulating inflammation and immune responses, for instance was AGP shown to reduce the phagocytic activity of neutrophils, superoxide anion production and migration(76). On the other hand AGP was also found to induce rises in intracellular Ca^{2+} via siglec binding in primed neutrophils (77). An elevation of the cytosolic concentration of free calcium ions is a common feature to the activation of the secretory system and exocytosis of intracellular vesicles (78).

Alpha-2-macroglobulin is a plasma protease inhibitor composed of two non-covalently associated dimers of disulfide linked identical subunits of ~180 kDa, and has a total of 32 N-glycosylation sites (8 per subunit). Within its sequence a proteolysis-sensitive region is found, which when cleaved by proteases leads to a conformational change that captures and inactivates the protease. Alpha-2-macroglobulin also has other functions and interactions with tissue cells. The conformational change when the bait-region is cleaved reveals a binding site for the N-glycosylated cell surface receptor LRP-1 (low density lipoprotein receptor related protein-1), a member of the LDL receptor gene family. The binding of alpha-2-macroglobulin to LRP-1 induced cell proliferation and MAP kinase activation in a macrophage derived cell line. Interestingly this was inhibited by LPS (79) which suggests that the macrophages were activated in a M2-polarized pathway since LPS activates macrophages in the classical (M1) way (80-82). Several growth factors have

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been reported to interact with alpha-2-macroglobulin (83). A consequence of this may be stabilization against renal filtration which was a suggested feature of alpha-2-macroglobulin binding TGF- β (83).

Haptoglobin consists of two different polypeptide chains, and occurs in different genetically determined forms in different individuals. The α -chain that Haptoglobin polymorphism arises from exists in two variants (9 and 16 kDa) and the β -chain (40 kDa) with 4 glycosylation sites (69, 84) causes further microheterogeneity. Individuals carrying the allele (either one or two) coding for the α^2 -chain will form haptoglobin polymers with up to 6 β -chains, thus forming highly glycosylated complexes (whereas individuals with the α^1 -chain only will form a complex including two β -chains). Haptoglobin has several functions, apart from the well known haemoglobin-binding and anti-oxidative roles, it has been identified as a galectin-3 ligand in sera of colon-cancer patients (25), an angiogenic factor in serum (85, 86), required for proliferation and differentiation of endothelial cells during formation of new blood vessels and having immunomodulatory effects (87). It has also been suggested to be involved in remodelling in asthma by inducing fibroblast differentiation (88). A range of studies indicate that haptoglobin levels may be raised and the N-glycan structures on the protein altered, having more fucosylation and more tri-, and tetra antennary structures in different cancer forms (60, 61, 70, 75).

Galectins and inflammation

Galectins are implicated in inflammation and fibrosis (89-91) and have been shown to modulate steps of the inflammatory response, such as cell-matrix interactions(92), cell trafficking, survival of cells (93) and secretion of cytokines and other inflammatory factors (14, 39, 45, 50, 94, 95). Below follows an

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overview over inflammatory steps and development of chronic inflammation and then further examples of galectin involvement in chronic inflammatory conditions.

General mechanisms of inflammation and development of chronic inflammation

The acute inflammation is rapid and usually becomes obvious within minutes after tissue trauma, the symptoms of redness, heat oedema, and pain that were described already 2000 years ago represent cooperation between vascular, neurological and cellular responses to the initial damage (96-98). Damage to blood vessels is an initial event of the damaged tissue and possibly also microbial invasion. The blood clotting system is activated, forming a clot that restores vessel integrity and creates a scaffold generated by activated fibrinogen that forms a three dimensional structure where platelets and blood cells are trapped. Aggregating blood cells release coagulation factors, leukocyte recruiting factors and growth factors that evoke a vascular response including changes in permeability, blood flow and adhesiveness. The influx of fluid increases the concentration of important serum proteins e.g. components of the complement system and immunoglobulins. The endothelial cells are directly involved in the inflammatory response by releasing coagulation and permeability factors, recruitment factors and proinflammatory cytokines such as tumour necrosis factor- α (TNF- α) and IL-1. When stimulated by, for example, TNF- α and IL-1, endothelial cells begin to express adhesion molecules on their luminal surface. The adhesion molecules can be divided into four families: the integrins, the intercellular and vascular adhesion molecules (ICAM and VCAM), the selectins and the mucin-like selectin receptors. When blood leukocytes passes by the site of injury the adhesion molecules causes them to halt. The combination of increased blood flow, vascular permeability and expression of adhesion molecules and chemoattractants by endothelial cells

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causes the leukocytes to leave the circulation and enter the tissue. The process of immune cell migration out of the blood vessel is divided into steps where the leukocytes first are rolling mediated by selectin-ligand interactions, then are induced by local chemokines to halt and adhere firmly, as mediated by integrin – ligand interactions, to the vessel wall and then extravasate into the tissue (4). When the leukocytes have bound the adhesion molecules, chemotactic signals from the inflammatory site allow them to migrate to the inflammatory site.

Inflammatory factors of the acute inflammation -The first cells to reach the site of damage are usually neutrophils which start to destroy microbial agents and phagocytose tissue debris (99). The factors released by the coagulation pathway and complement activation together with serum and cell derived products initiate and amplify the inflammation. Additionally, platelets, endothelial cells, leukocytes and the surrounding connective tissue-cells contribute to the inflammation by producing inflammatory factors. Leukocytes produce cytokines, enzymes and lipid mediators that orchestrate the inflammatory response. Phospholipases are activated to degrade membrane phospholipids, this initiates the cyclooxygenase pathway that produces prostaglandins and eventually leukotrienes. As the neutrophils are clearing the area, monocytes enter the inflamed tissue by a similar pathway. Additionally, nearby tissue monocyte-derived macrophages arrive, together with neutrophils they cause macrophages to increase the clearance of damaged tissue and microorganisms (100). Macrophages have a broad range of functions: they can destroy and phagocytose bacteria and tumor cells but also regulate the inflammatory response, they produce cytokine, growth factors and matrix-inducing molecules. Macrophages are involved in the initial phase of inflammation where classically activated (M1-polarized) macrophages kill and remove bacteria. These, cytotoxic and proinflammatory activities of M1 macrophages, are enhanced by Th1-cytokines as IFN- γ or IL-12. M1-polarized activation of macrophages is also associated with the proinflammatory cytokines

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TNF- α and IL-6 (80) that induce a cascade of other cytokines. After antigen presenting cells have become activated they travel from the tissue to a lymph node, there they encounter and prime lymphocytes and initiate different immune responses (101). The lymphocytes in turn function in activation of macrophages and are also central for the B-cell antibody production (CD4+ T-cells) or function in direct killing of cells through antigen recognition (CD8+ T-cells).

In the resolution of inflammation microbes or other inflammatory stimuli are cleared damaged tissue removed. Vascular integrity is restored, damaged tissue replaced and if needed, the epithelium resurfaces the former inflammatory site.

Cytokines that supported initiation and maintenance of inflammation are also involved in the resolution by inhibition of the inflammatory response. IL-4 is an anti-inflammatory cytokine that inhibits neutrophil production of IL-8, reducing procoagulant activity of endothelial cells (102) and blocking macrophage activation and promoting their apoptosis. IL-4 also induces differentiation of T-lymphocytes into T-helper cells type 2 (Th2) that activates M2 polarized macrophages (80). The macrophages contribute to the proliferative phase by synthesizing e.g. growth factors e.g. FGF necessary for the tissue regeneration. In the formation of new tissue, angiogenetic mechanisms are necessary, e.g. enzymatic degradation of the basement membrane and extracellular matrix, migration and proliferation of endothelial cells forming a tube stabilized by mesenchymal cells and matrix. Eventually growth is inhibited by interferons and other cytokines.

Chronic inflammation -If the inflammatory stimuli fail to be removed, the initiating phases of inflammation will not go down but become chronic and occur at the same time as tissue remodelling and healing (103, 104). The chronic inflammation can be caused by irritants first evoking an acute inflammation that then becomes chronic when the immune system fails to eliminate the irritant

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(e.g. in tuberculosis), self antigens that cause an autoimmune response (e.g. in rheumatoid arthritis) or a low intensity irritant that persists over a long time period but does not necessarily cause an acute reaction (e.g. in tobacco smoking). While an acute inflammation usually has resolved after three weeks, chronic inflammation can subjectively be defined as lasting for more than six weeks. The persistent inflammation causes significant tissue destruction.

In chronic inflammation cells as macrophages and lymphocytes dominate. The M2-polarized macrophages of the healthy resolution phase of inflammation have also been implicated in various events of chronic inflammation e.g. asthma, fibrosis and atheromatous plaques (80) and contribute by producing proinflammatory cytokines as IL-1, TNF- α and IL-6 (105). Also non-immune cells are affected in chronic inflammation and contribute to unwanted “tissue remodelling”. For example, the differentiation of fibroblasts into myofibroblasts contributes to development of fibrosis.

Asthma

Asthma is one example of airway chronic inflammation where particularly mast cells, eosinophils and T-lymphocytes are important for disease development (106-109) but also neutrophils (110). Symptoms are usually associated with limitation of airflow that is partly reversible. The chronic underlying inflammation of airways is linked to the bronchial hyperresponsiveness to nonspecific triggers as allergens, exercise, cold air etc. Already in mild asthma certain characteristics as mucus hypersecretion and goblet cell hyperplasia, oedema of airways, epithelial shedding, smooth muscle hypertrophy, infiltration of eosinophils in the bronchial wall, activation of mast cells and deposition of collagen under the basement membrane can be observed.

Allergic diseases are associated with Th2-phenotype T-cells. T-lymphocytes interact with B-lymphocytes to regulate the production of allergen specific IgE. Epitopes on allergens are recognized by dendritic cells and

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processed fragments are presented to T-cells via the T-cell receptor and MHC-II on the antigen presenting cell. Costimulatory molecules as adhesion molecules are also needed. In the presence of IL-4, T-cells differentiate to Th2-cells that in turn express IL-4 and IL-13, their action on B-cells cause isotype switching from IgM and IgG to IgE. Cross-linking of IgE molecules and IgE receptors on mast cells leads to release of a range of inflammatory mediators e.g. histamine, heparin and tryptase. Other cells with IgE receptors are basophils, dendritic cells, eosinophils, macrophages and platelets.

Eosinophil recruitment is regulated for example by activated mast cells and T-cells. To cross the vascular epithelium and enter the airways, cell adhesion molecules (CAMs) are needed. These are expressed on leukocytes and endothelial cells. Glycan-structures e.g. Sialyl Lewis X on eosinophils interact loosely with lectin-binding regions of P-, L-, and E-selectins, making the leukocyte roll along the endothelial cells. The selectin expression is upregulated by e.g. histamine, leukotrienes, IL-1 and TNF- α . The rolling along the endothelium is arrested when integrins on the leukocyte interact with ICAM-1 on endothelial cells that begin to migrate into the tissue.

Airway remodelling of asthma -The loss of bronchial epithelium and its ciliary layer that occurs in the airways of asthma patients impairs mucus clearance and allows better entrance for allergens and noxious stimuli to smooth muscle cells and sensory nerve receptors of the bronchial tissue (111). This initiates a repair response that leads to remodelling, where e.g. platelet-derived growth factor and basic fibroblast growth factor induces myofibroblasts to proliferate. In the presence of TGF- β , the myofibroblasts start to synthesize collagen and other matrix molecules as β -laminin and versican, which thickens the basement membrane. These proliferative and repair processes are part of the pathology of chronic inflammation in asthma and may continue to involve nerves, blood vessels, and smooth muscle together with matrix deposition in submucosa and adventitia.

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Involvement of galectins in asthma and other chronic inflammatory conditions

Galectins have been implicated in both inflammatory and tumorigenic processes (9, 14, 20, 28, 37-39, 45, 50, 93, 112, 113). Recently a rate limiting role for galectin-3 in chronic inflammation with fibrosis has emerged, based on its effect on alternative macrophage activation (M2-polarized) and fibroblast to myofibroblast differentiation (80, 91). In vivo studies in an asthma model revealed that galectin-3 was upregulated in peribronchial inflammatory cells and BAL and was necessary for development of lower airway hyperresponsiveness (27). In a model of renal fibrosis, galectin-3 secreting macrophages were shown to be necessary for the fibrotic development (89, 90). Another effect of galectin-3 is to promote T-lymphocyte anergy by distancing the T-cell receptor from CD8 (114).

Galectin-1 has been proposed to induce apoptosis in certain activated T-cells (Th1 and Th17), and thereby be able to modulate the balance in favour of Th2 (37, 40). The Th2-cells appears to be saved because of increased expression of terminal NeuAc linked to the 6 position of galactose, which as mentioned above would block galectin binding. Galectin-1 also has a possible inhibitory effect on recruitment of neutrophils for extravasation (115).

Other galectins also have immunomodulatory effects, although less studied than for galectins-1 and -3. Galectin-8 has been reported to bind to CD44 on neutrophils (116) and mediate adhesion via integrins on neutrophils (117) and T-cells (92) indicating a role in inflammation. Galectin-9 is secreted by T-cell derived cell lines (118) and interacts with TIM-3 involved in the function of regulatory T-cells.

Chronic inflammation, cancer and galectins

Reports have found galectins expressed in tumors but also in the surrounding stroma, indicating a cross-talk that is beneficial for the development of the tumor (8, 10, 119-122). Galectin-3 is expressed in tumor infiltrating macrophages (123), further indicating a direct connection between the roles of galectins in cancer and inflammation.

Chronic inflammation supports cancer

For expansion of a neoplastic tissue, tumor cells need to acquire unlimited self renewing capacity, but they also need to utilize cellular programs of the tissue that e.g. enhance blood flow, oxygenation and angiogenesis, additionally molecular programs are needed that support tumor cell survival and enhanced metabolism of the extracellular matrix favouring remodelling and expansion of the tissue (124, 125). Although a vast amount of genetic alterations are identified only a small number of cancers are caused by known cancer-susceptibility genes (126). Lately it has been discussed that the tumor is dependent of surrounding tissue for its development, where the mesenchyme surrounding the tumor undergoes inflammatory changes that actually supports the tumor e.g. by an increased angiogenesis (127). The processes of angiogenesis, cell migration, and remodelling of matrix are not unique to cancer but programs that are normally activated during development or in adult tissue as response to acute stress (128) the surrounding tissue is thus necessary to support the progression of cancer. This is supported by the fact that the cellular composition of cancers contains not only neoplastic cells but also genetically stable cells that are activated or recruited to the local microenvironment e.g. innate (82, 105, 124) or adaptive immune cells, cells of the vasculature or fibroblasts and other mesenchymal support cells. These cells contribute by releasing soluble mediators e.g. TNF, interleukins and interferons that regulate

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cell proliferation, migration, angiogenesis, tissue remodelling, metabolism and the integrity of the genome. This contributes to the increased risk of developing cancer that individuals suffering from chronic inflammatory disorders harbour.

Changes of glycosylation in cancer

The cell surface carries a large amount of N-glycosylated proteins. Glycoproteins can have many glycosylation sites which may be differently processed (129). It was found that when cells lack a glycosyltransferase (Mgat5) that catalyses addition of GlcNAc to form N-glycan tri- and tetra antennary structures, fewer tumors with poorer metastatic ability were formed. N-glycan branching of different proteins have been reported to be increased in a range of different cancers (24, 29, 70, 130-136) promoting the survival of the tumor (137) and metastasis (138). On the other hand certain cancer associated metalloproteinases showed decreased binding to galectin-3 indicating an increased range of activity (36).

Reported roles of galectins

Many growth factor receptors such as EGFR, PDGFR, FGFR and IGFR have several N-glycan sites, galectin-3 increases the time the growth receptor spends on the cell surface before it is recycled by connecting glycoproteins in a lattice (30, 136, 139). The galectin lattice competes with oligomerized caveolin-1 microdomains for epidermal growth factor receptor (EGFR). The binding of EGFR to the galectin-lattice is dependent of branched N-glycans. EGFR association with the galectin lattice reduces the EGFR diffusion rates and promotes receptor interaction with the actin cytoskeleton. EGFR association with the lattice also opposes sequestration by caveolin-1, overriding its negative regulation of EGFR diffusion and signalling (135) and decreases the time the receptor spends in endosomes.

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Galectin-8 was reported to mediate cell-adhesion via integrins (140), galectin-3 has also been suggested to be involved in adhesion and spreading of breast carcinoma cells. When cells were detached they rapidly began to secrete galectin-3, which was also taken up. The adhesion plaques of galectin-3 containing cells had a different appearance than control cells and it was suggested that the plaques were better suited for rapid adhesion and spreading as opposed to those of control cells that seemed to be suited for firmer adhesion (21).

Levels of galectin-1 and -3 have been reported to be elevated in patients suffering from head and neck squamous cell carcinomas (HNSCCs) (141), as well as in breast cancer cells (142). Additionally, galectin-1 expression was increased in breast cancer associated stroma and this could be correlated to tumor invasiveness (143). Galectin-1 is expressed in endothelial cells of activated lymphoid tissue and was also observed in endothelial cells of e.g. colon carcinoma (44).

PRESENT INVESTIGATION

Aim

As exemplified above, galectins have been implicated in cancer and inflammation based on a variety of effects in cell culture and *in vivo* using e.g. null mutant mice. Even if the implications appear convincing, it remains more unclear how the galectins actually act at the molecular level in relevant cellular systems; effects on cell migration, adhesion and retaining growth factor receptors on the cell surface are some suggestions. The aim of the present study was to shed further light on this, by analyzing how galectins recognize carbohydrates in their natural context, as exemplified by glycoproteins in serum and in airway tissue fluid. Paper I first gives an overview of ligands for different galectins in serum of healthy persons. Paper II focuses on whether there is a difference in the amount of galectin-1 ligands in sera of breast cancer patients compared to healthy persons, and paper III identifies and compares ligands of galectins-3 and -8 in bronchoalveolar lavage from asthma patients and healthy individuals.

Identification of serum glycoproteins from healthy persons that interact with galectins (Paper I)

The start point of paper I was the already known sugar structures that galectins prefer. The aim was to identify natural ligands of galectins in serum of healthy individuals and thereby find out more about functions and differences concerning natural ligands among the galectins. Serum is an easily obtained bio-fluid, commonly stored in bio-banks, it contains a wide array of glycoproteins and since tissue proteins diffuse into serum it contains a broad spectrum of functionally important proteins. Serum proteins diffuses out in tissues and can be found there at approximately 50% of the concentration in blood (144), thus

PRESENT INVESTIGATION

there are high concentrations of serum glycoproteins in tissues that galectins may interact with. Serum was applied to affinity chromatography on immobilized galectin-1, -2, -3, -4, -7, -8 or -9 and bound proteins identified using mass spectrometry.

It was clear that galectins bind serum glycoproteins very differently, galectin-3, -8 and -9 bound several different serum glycoproteins, and galectin-1 bound some, whereas galectin-2, 4 and 7 do not bind the kind of glycoproteins found in serum. This was confirmed by the fact that fluorescein tagged saccharide probes still bound the galectins even if serum glycoproteins did not. Using a mutant galectin-3 that lacked binding capacity for LacNAc-moieties as a negative control, we confirmed that the serum glycoprotein interaction was not unspecific. The identified galectin ligands differed among galectins, with galectin-3 as the “best” binding galectin, galectin-8 and-9 bound similarly as galectin-3 but some proteins differed among the galectins. Alpha-2-macroglobulin and haptoglobin were the most commonly found ligands, others were transferrin and alpha-1-acid glycoprotein.

Galectin-1 binds more glycoproteins from sera of cancer patients compared to healthy individuals (paper II)

The aim of paper II was to determine whether there would be more galectin-1 binding proteins in sera of breast cancer patients compared to healthy individuals. Expression of galectin-1 in tumors and surrounding stroma has been correlated to faster progression and worse outcome. Several previous reports have stated that changes of glycosylation occur in various cancer forms, this combined with the fact that some acute phase proteins rise during the progression of cancer made it likely that more proteins would bind galectin-1 in cancer sera. Additionally, galectin-1 only binds intermediate serum glycoprotein levels in healthy persons, compared to the other investigated galectins in paper I which may be an advantage when detecting increased binding.

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We found that galectin-1 bound approximately double the amount of serum proteins to that of healthy persons. The major ligand was alpha-2-macroglobulin in healthy persons, while haptoglobin was the protein that increased most in cancer patients.

Identification of galectin-3 and -8 ligands in bronchoalveolar lavage (paper III)

In paper III the aim was to study whether galectin-3 and -8 bind different and/or more proteins in a state of chronic inflammation compared to a healthy condition. Additionally, we compared the identified ligands of galectin-3 and galectin-8. Galectin-3 is implicated in the alternative activation of macrophages that occurs in asthma and other conditions of chronic inflammation and galectin-8 was reported to mediate neutrophil adhesion via integrins, these made them both interesting targets for investigation. The epithelial lining fluid that is a part of BAL reflects the factors that affect the lung and can thus already in mild asthmatics be altered.

The ligands differed between the galectins, additionally some ligands were found only in asthma patients, this was the case for macrophage mannose receptor and CD59 as galectin-3 ligands and e.g. haptoglobin, CD55 and macrophage mannose receptor as galectin-8 ligands. Of the identified ligands haptoglobin has already been implicated in the differentiation of fibroblasts into myofibroblasts (88).

For one of the ligands, endocytosis was studied. Transferrin was endocytosed together with galectin-3 in fibroblasts obtained from the airways (Fig. 6). At the cell surface transferrin co-localized with galectin-3, but after endocytosis galectin-3 went back to the cell surface while transferrin remained inside the cell.

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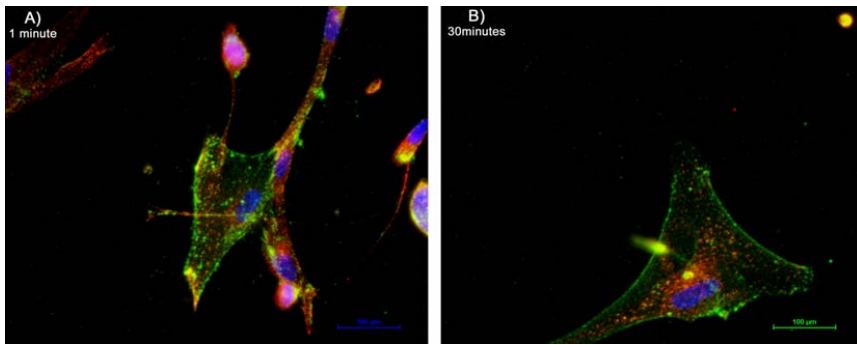


Fig 6. Fibroblasts were incubated with galectin-3 (green) and transferrin (red). A) After 1 minute galectin-3 and transferrin colocalize (yellow) at the cell surface and galectin-3 is endocytosed together with transferrin. B) After 30 minutes galectin-3 has recirculated to the cell surface while transferrin is localized inside the cell.

Summary and comments on methods used

Analysing bronchoalveolar lavage (BAL)

While serum is easy to obtain, samples of tissue fluids are more difficult. The airways and the alveoli foremost are covered with a thin layer of epithelial lining fluid which is a source of different cell types and soluble components of the lung that protects it from damage and preserve the gas-exchange capacity. Bronchoalveolar lavage (BAL) is the most common way of sampling epithelial lining fluid ([145](#), [146](#)). BAL will typically contain cells, lipids, nucleic acids, proteins and peptides ([147](#), [148](#)). In a healthy individual the cellular content mainly consists of alveolar macrophages (that correspond to 80-95% of the cellular content) lymphocytes, neutrophils, eosinophils and sometimes plasma cells. Phospholipids that decrease alveolar surface tension are synthesized by pneumocytes ([149](#)) and constitute the main component of the surfactant. The epithelial lining fluid reflects the external factors that affect the lung. This can be used for early diagnosis and identification of disease markers. Specific patterns of protein expression from biofluids such as BAL and serum, as

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discussed in the background, can be used as prognostic and diagnostic measurements of disease status ([150](#), [151](#))

Affinity chromatography

The main method to collect galectin ligands for further identification was affinity chromatography (Fig. 7). Affinity chromatography separates proteins on the basis of their ability to reversibly interact with a specific ligand/receptor (in this case the galectin) coupled to a chromatography matrix ([152](#)). Affinity chromatography is unique since it purifies molecules based on the biological binding activity of the protein. Since galectins interact with galactose-moieties on glycoproteins, bound glycoprotein-ligands are easily eluted from immobilized galectin by using lactose as a competitive ligand.

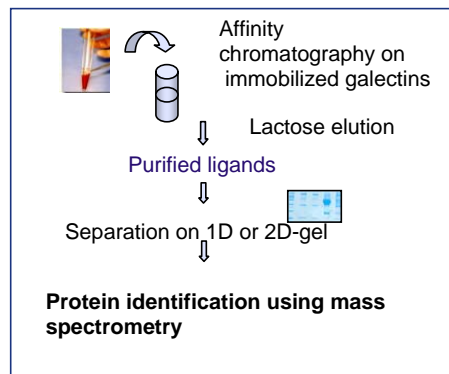


Fig 7. Experimental setup: Proteins interacting with galectins can be identified by passing the bio-fluid of interest over galectins immobilized on a chromatography matrix. Non-interacting proteins are washed off while interacting glycoproteins can be purified and eluted using lactose as a competitive ligand. The purified galectin binding proteins may be analyzed using separation on a SDS-PAGE gel and immunoblotting, by direct analysis using shot gun proteomics, or as shown in figure mass spectrometry identification after separation on SDS-PAGE.

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Mass spectrometry

For identification of the galectin bound glycoproteins isolated by affinity chromatography, mass spectrometry was used. After the protein had been digested with trypsin, the mass of the resulting peptides was determined by mass spectrometry. In this technique, the peptides are first ionized and then their mass-to-charge ratio (m/z) determined, from which the mass can be calculated. The number of ions at each mass-to-charge value is registered by a detector. Different techniques can be used for ionization, e.g. matrix-assisted-laser-desorption ionization (MALDI) or electrospray ionization (ESI), and mass analysis, e.g. time-of-flight (TOF), and Fourier transform ion cyclotron resonance (FTICR) (153). In the present case MALDI-TOF was used to analyze semipurified proteins from bands cut from SDS-PAGE gels. Liquid chromatography coupled to ESI-FTICR was used to analyze peptides from the total mixture affinity isolated proteins (154). This is possible, because the latter technique also permitted a second step of fragmentation and mass spectrometry of each peptide, to determine its sequence. The peptide masses and/or sequences could then be compared to large data bases of known proteins, from which the likely identity of the original protein could be obtained. The computer systems for this proteomics analysis (155-158) is now very well developed, as described in the Methods sections of the paper I and III. Glycan structures were also analyzed in paper I.

DISCUSSION

By this thesis we wanted to clarify what glycoproteins galectins bind in bio-fluids as serum and BAL in an effort to better understand the elusive functions of galectins. We found that some galectins bind much more serum glycoproteins than was previously known, this tells us that these galectins are more or less always surrounded by ligands. Furthermore, the amount of serum glycoproteins that galectin-1 binds is increased in breast cancer patients to double that of healthy individuals, this increase is mainly caused by haptoglobin and may reflect increased mechanisms of angiogenesis (85, 86). This may lead to new screening methods for earlier detection of breast cancer. Additionally we found that galectin-3 and -8 binds different sets of glycoproteins in asthma patients compared to healthy individuals and thus are mediating effects of other proteins than they normally are. We report that some galectins, widely expressed as galectin-3, -8 and -9 bind serum glycoproteins well, whereas galectin-1 binds intermediate levels and galectin-2, -4 and -7 that have a more restricted, tissue-specific expression bind serum glycoproteins poorly. One reason for this might be that galectins with a more restrictive tissue expression are adapted to the specific glycoproteins of that tissue e.g. are glycoproteins in the gastrointestinal tract more fucosylated than in serum and galectin-4 that is expressed in the GI-tract has affinity for fucosylated-GalNAc-ligands.

The serum glycoproteins that galectins bind are immunomodulatory glycoproteins, with e.g. anti-oxidative effects that might be beneficial for the cell. Some of the bound proteins are also involved in delivery of iron as the haptoglobin-haemoglobin-complex, transferrin, hemopexin, or in delivery of lipids like apolipoproteins. It might be advantageous for a cell to bind certain proteins to the cell surface e.g. proteins with antioxidative properties that may

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protect the cell-surface especially in an inflammatory environment as immune cells are, or tumor cells that usually evoke a response of chronic inflammation in the surrounding mesenchyme.

The binding of serum proteins depend to a large extent on the number of N-glycan sites on the protein and number of available antenna for galectin binding. Galectins with a more modest serum glycoprotein binding ability like galectin-1, bound proteins as haptoglobin, alpha-2-macroglobulin and Igs that contain several glycosylation sites. Galectin-3, in comparison, was the galectin that bound most types of serum glycoproteins. Gal-3 binds part of transferrin (~15% that have glycan structures available for gal-3 binding).

The glycoprotein-galectin lattice on cell surface, discussed in the background is important for the time a glycoprotein spends on the cell surface. Since galectin-3 binds several serum glycoproteins e.g. transferrin (paper I) and also its receptor (unpublished), galectin-3 may be involved in (1) connecting the ligand and the receptor, (2) in receptor-ligand trafficking after the ligand has bound its receptor on the cell surface, and/or (3) involved in functions the individual glycoproteins mediate e.g. differentiation, cell migration of fibroblasts or growth enhancement. High hexosamine levels, the building blocks of glycans, affect the branching of N-glycoproteins ([29](#), [30](#)), making them more available to galectins. This will affect low N-glycan proteins by suddenly making them available to galectins.

Even though galectin-3 is widely expressed in the body all cell types may not secrete it, macrophages does however ([89](#)) and may contribute to the extracellular, cell surface and subsequently intracellular levels of galectin-3 in cells around an inflammatory site.

In paper II, we chose to study whether this might be of importance in a pathological condition by comparing the amount of bound proteins between healthy persons and breast cancer patients. Since it might be difficult to detect a binding difference of a galectin that already binds well in a healthy condition as

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galectin-3 does, we looked at the intermediate binding galectin-1. It was shown to bind more glycoproteins from sera of breast cancer patients than from healthy individuals, reflecting either an increase in haptoglobin levels, a change of glycosylation, or a combination of both. Cytokines, inflammation and cancer are known to cause both increases in acute phase proteins and glycosylation changes. Since common serum proteins bound by galectins, e.g. haptoglobin is known to induce angiogenesis and other bound proteins having other beneficial characteristics, as discussed above, may be beneficial to the tumor and may aid in its survival and growth.

As discussed further below, it is likely that glycosylation changes occur in pathological conditions that affects the binding of galectins. In this paper the approach was to determine that galectin-1 binds more serum glycoproteins in the sera of breast cancer patients which might make it a useful tool of detecting cancer.

In paper III we used the powerful LC-MS/MS mass spectrometry technique to identify and compare galectin-ligand profiles in BAL. Galectin-3 and galectin-8 are two galectins expressed in lungs and with implications in inflammatory mechanisms. They both bind readily detectable levels of serum proteins and are hence good candidates for studying the low protein-concentration-fluid that BAL is and for identifying interaction partners in asthmatic patients. Galectin-3 and -8 binding proteins from bronchoalveolar lavage were identified showing, that apart from the serum glycoproteins identified in paper I, galectins bind proteins specific to the lung. In the mixture of glycoproteins as serum and BAL constitute, galectin-3 and galectin-8 that both have high preference for serum glycoproteins bind different sets. This becomes more obvious when functionally grouping the bound proteins. Although many of the bound proteins overlap, the profiles of galectin-3 and -8 bound proteins are distinct. Since galectin-3 and -8 bind different proteins, it is

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likely that they may mediate different effects. When comparing the asthma patients with the healthy individuals, profiles are changed both for galectin-3 and -8, i.e. in the BAL of asthma patients, galectins bind other glycoproteins than in healthy individuals. Furthermore, glycosylation must be changed already in the early pathological condition that mild asthma constitutes, because compared to a non-asthmatic state proteins e.g. transferrin was identified as a galectin-3 binding protein both in asthma patients and healthy individuals whereas (using BAL from the same individuals) only transferrin from asthma patients bound galectin-8, i.e. the glycosylation of transferrin has changed from being preferred by galectin-3 to being preferred by both galectin-3 and -8 in the pathological condition.

One of the identified galectin-3 ligands in BAL: macrophage mannose receptor, was only identified in asthma patients. In murine macrophages, macrophage mannose receptor was found to be upregulated after treatment with the Th2 cytokines IL-4 or IL-13 that drives the M2 polarized activation of macrophages implicated in pathologies of fibrosis in e.g. asthma (80, 89). Thus, the connection between galectin-3 and fibrosis is further strengthened by implicating that gal-3 work through the macrophage mannose receptor in the pathology of fibrosis.

The conclusion that glycosylation is changed in asthma patients compared to the normal state is supported by literature concerning both glycosylation in acute and chronic inflammation (60, 74, 75). Galectin-8 and galectin-3 are both expressed in the lung although they are in different areas. It seems, by looking at the ligands of galectin-3 and -8 that the galectins have distinct functions. In the bronchoalveolar environment of chronic inflammation that asthma patients acquired, the profile of galectin-3 and galectin-8 binding proteins have been altered compared to the healthy subjects but the ligand profiles are still not the same between the two different galectins (17, 117, 140, 159, 160).

FUTURE PERSPECTIVES

Since several galectins bind such a wide range of common glycoproteins, it seems important to take this into account when studying galectin functions. Questions raised are: Is the combination of proteins that bind cells important? Are glycosylation changes in individual glycoproteins competing for galectin binding and thus affecting the combination of bound proteins in e.g. a galectin-glycoprotein lattice on the cell surface? The amount of a specific glycoprotein, its number of N-glycans and how many of these that are available for galectin binding together with how many other glycoproteins that are available, are things that have to be considered when studying a galectin-glycoprotein interaction and effects galectins exert. Since serum glycoproteins diffuse out in tissue to approximately half the concentration of that in serum (depending of size of the protein) galectins are most likely always surrounded by a high concentration of serum ligands that will compete with binding to other ligands.

Both the galectin expression of a cell and the glycoprotein composition around the cell (e.g. induced by inflammation) can be altered (91), this may result in a new message to the cell (as discussed in the background, many common serum proteins have more complex roles than generally recognized) delivered through the cell surface galectin-glycoprotein lattice.

As mentioned in the discussion the glycosylation of BAL-proteins is likely to be altered in asthma patients and galectin-1 bound more glycoproteins in sera from breast cancer patients than of healthy individuals, thus galectins might be useful as tools for detection of pathological changes of glycosylation. Both galectin-3 and galectin-8 were found to bind some proteins only in BAL of asthma patients (e.g. the macrophage mannose receptor), this might be useful for clarifying functions of galectins and pathological mechanisms but may also serve as biomarkers of disease. Additionally galectins may be useful in

FUTURE PERSPECTIVES

identification of novel biomarkers as they sort out non-glycosylated proteins as albumin and prefer branched N-glycans that are increased in cancer and chronic inflammation (70).

POPULÄRVETENSKAPLIG SAMMANFATTNING

Galektiner är socker-bindande proteiner, de finns inuti och utanpå kroppens celler och de kan påverka sjukdomstillstånd som inflammation och cancer. Det finns kopplingar mellan dem och de mekanismer som styr kronisk inflammation och cancer bla en ökad cellförnyelse som normalt skulle lett till läkning, men istället kan dessa processer leda till att cancerceller kan föröka sig.

I den här studien har vi undersökt hur galektiner är kopplade till kronisk inflammation och cancer genom att undersöka vilka proteiner i kroppsvätskor de binder, först hos friska människor sedan cancer patienter och till sist astmatiker som har en mild form av kronisk inflammation. I den första delen av studien där friska människor studerades fann vi att det varierar mellan olika galektiner hur väl de binder sockerstrukturer på proteiner i blodomloppet. De sorters galektiner som finns i stora delar av kroppen binder många olika sorters proteiner i blodplasma medan de galektiner som uttrycks på få ställen i kroppen nästan inte binder dem alls. Därefter undersökte vi om det är någon skillnad mellan hur väl galektiner binder plasmaproteiner hos friska personer jämfört med bröstcancer patienter. Vi fann att ett galektin (galektin-1) band ungefär dubbelt så mycket proteiner hos cancersjuka vilket antagligen beror på att det finns mer av vissa proteiner och att sockerstrukturerna på dessa proteiner är förändrade så att galektiner binder bättre till dem.

I skölvätska från friska människors och astmatikers luftvägar fann vi att olika galektiner inte binder till samma proteiner vilket antagligen beror på att galektinerna inte medverkar i exakt samma biologiska skeenden. Vidare fann vi att de inte heller binder samma proteiner hos friska personer som de gör hos astmatiker, det betyder troligen att sockerstrukturerna på proteinerna är förändrade även i detta sjukdomstillstånd och att galektiner medverkar i andra biologiska skeenden i astmatikers luftvägar än de gör i friska människors.

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-Cecilia Cederfur, April 28, 2008

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