Lectin histochemistry: historical perspectives, state of the art and future directions

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To be published in:

Histochemistry of single molecules: Methods and protocols [ISBN: 9781071626740] / edited by Carlo Pellicciari, Marco Biggiogera, Manuela Malatesta (Springer, 2022).

CONTENTS

i Abstract

Lectins, discovered more than 100 years ago and defined by their ability to selectively recognise specific carbohydrate structures, are ubiquitous in living organisms. Their precise functions are as yet under-explored and incompletely understood but they are clearly involved, through recognition of their binding partners, in a myriad of biological mechanisms involved in cell identity, adhesion, signalling and growth regulation in health and disease. Understanding the complex 'sugar code' represented by the 'glycome' is a major challenge and at the forefront of current biological research. Lectins have been widely employed in histochemical studies to map glycosylation in cells and tissues. Here, a brief history of the discovery of lectins and early developments in their use is presented along with a selection of some of the most interesting and significant discoveries to emerge from use of lectin histochemistry. Further, an evaluation of the next generation of lectin-based technologies is presented, including the potential for designing recombinant lectins with more precisely defined binding characteristics, linking lectin-based studies with other technologies to answer fundamental questions in glycobiology, and approaches to exploring the interactions of lectins with their binding partners in more detail.

ii Keywords

lectin histochemistry, history, carbohydrates, glycosylation, review, methodology, glycomics

1 Historical perspectives

1.1 What are lectins?

The term 'lectin', first suggested by Boyd and Shapleigh in 1954 ***(1)***, is derived from the Latin *legere* which means 'to select or to choose'. It was originally applied to a group of proteins, mainly derived from plants, that had the property of being able to bind to specific carbohydrates and which agglutinated cells. Subsequently, lectins have been described from a diversity of biological sources as wide ranging as mammals, slime moulds, fungi, invertebrates and bacteria and are recognised as being ubiquitous in living organisms. They are very heterogeneous in structure, usually multivalent, and are distinct from immunoglobulins that recognise carbohydrate-based antigens, or enzymes that have carbohydrates as their substrate ***(2)***. Their precise function within the organism that produces them is often largely unknown, but it is increasingly being recognised that lectin-carbohydrate binding interactions are functional in a diversity of biological processes involving cell-cell recognition, adhesion and signalling and that this is an area of research that is under-explored and potentially extremely revealing.

1. 2 Lectins – a brief historical introduction

Lectins initially aroused interest at the end of the 19th century and during the early years for the 20th century and were first described during investigations into toxic plant extracts that were used in medicine at that time. During this period, the ability of 'hemagglutinins', as they were known initially, to selectively agglutinate cells and precipitate glycoconjugates and polysaccharides was established, as was the

inhibition of these reactions by certain carbohydrates and carbohydrate-containing substances.

The lectin 'story', which is well reviewed by ***(3,4)***, begins with Stillmark who in 1888 and 1889 purified a protein from a toxic extract of *Ricinus communis*, castor oil, seeds which he named 'ricin'. He tested its effects upon various different cells and noted that sometimes he saw an agglutination reaction 'like in clotting'. Meanwhile, descriptions of toxic extracts from other plants including *Abrus precatorius*, the jequirty bean (named 'abrin') and *Robinia pseudoacacia*, the black locust (named 'robin') that could also agglutinate cells emerged. Over the twenty years following Stillmark's original description of ricin, a great many studies were published describing dozens of new hemagglutinins from diverse sources. Owing to ease of availability, most were derived from plants and it became apparent that seeds of the *Leguminosaeae, Euphorbiaceae* and *Solanaceae* were particularly rich sources. However, lectins were also described in fungi, invertebrates, and the venom of snakes. Over time, it became clear that lectins are ubiquitous in nature.

As described by ***(3,4)***, Stillmark, in his original description in 1889, noted that the presence of serum effectively inhibited the agglutination of erythrocytes by ricin, and later workers also noted that various biological substances such as gastric mucus could sometimes inhibit or reverse agglutination. At the time, the significance of these observations was not realised, but they were the first indications of the carbohydrate-binding properties of lectins. Watkins and Morgan (1952) ***(5)*** were the first to show that simple sugars (monosaccharides) are able to inhibit lectin binding.

While there were reports from the early years of the 20th century that 'hemagglutinins' did not always agglutinate erythrocytes from different human individuals to an equal extent, the ability of some lectins to distinguish between erythrocytes of different blood groups was not realised until in 1949 Boyd and Reguera ***(6)*** reported that an extract from *Phaseolus limensis*, Lima beans, agglutinated human blood group A erythrocytes, but not blood group B or O cells**.** An enormous amount of interest in the search for blood group-specific lectins followed, and the literature of the 1950's and 1960's reports the screening of hundreds of plant extracts, in particular, although extracts from other, often exotic, sources, for example, fish sera and snake venom, were also tested. By 1964, more than 100 blood group-specific lectins had been described. Morgan and Watkins (1959) ***(7)*** were first to demonstrate that the blood group specificity of lectins resulted from their carbohydrate-binding preferences. They showed that agglutination by the blood group A specific lectin from *Phaseolus limensis* could be inhibited by the presence of N-acetyl-D-galactosamine (GalNAc), whilst agglutination by *Lotus tetragonalobus* lectin, specific for blood group O erythrocytes, could be inhibited by alpha-methyl-L-fucose (Fuc). This is some of the first evidence of the presence of carbohydrates on the cell surface.

The first mammalian lectin to be described was the galactose (Gal)-binding liver lectin which is responsible for uptake and subsequent degradation of asialylated glycoproteins ***(8)***, and this was followed by the first description of a member of the galactoside-binding lectin family, the galectins ***(9)***. Perhaps one of the best studied families of mammalian lectins are the selectins, whose role in lymphocyte migration

and homing in immune surveillance is understood in some detail (see ***(10)*** for review). Moreover, lectin-carbohydrate recognition is a fundamental element of the mammalian innate immune response with, for example, a mannose (Man)-binding lectin functioning in pathogen recognition and initiating the complement cascade (reviewed by ***(11)***). It is now well established that lectin-carbohydrate recognition is a fundamental signalling and recognition system in virtually all living things, including humans.

Investigations into the functions of lectin-glycan binding interactions *in vivo* have been facilitated too by the development of lectin-deficient animals. This originated in the mid 1990's when advances in gene technology enabled the development of mice deficient in P-selectin ***(12)***, E-selectin ***(13)***, and both E- and P-selectin ***(14)***, and revealed the functions of these selectins in leukocyte trafficking and extravasation at sites of infection. Later studies (for example ***(15)***) have further revealed the function of selectins in cancer cell interactions with the endothelium during metastasis. Since that time, a number of lectin-deficient animals have been developed including, for example, those lacking mannose-binding lectin, the Lewis sugar-binding C-type lectin SIGNR1, and several members of the galectin family, and have been instrumental in our understanding of the functional roles of these lectins *in vivo*.

2 Lectins as tools for histochemistry

2.1 The development of lectin histochemistry

During the 1980's the technique of immunohistochemistry gained great popularity and was widely employed to study the distribution of biomarkers in normal and

diseased tissues. Immunohistochemistry uses antibodies as reagents to detect the cell or tissue localisation of a specific antigen through identification by a label that can be identified using microscopy. It originated in the 1940's and 1950's when Coons and colleagues ***(16-18)*** employed directly fluorescently-labelled antibodies to localise antigens in tissue sections using microscopy. Significant developments then emerged, including the use of a range of fluorescent, enzyme-based, radioactive and electron-dense labels, and various direct and indirect detection systems to enhance specificity and sensitivity (see ***(19)*** for review). It was soon realised that the basic concept underlying immunohistochemistry, that of detecting the binding of an antibody directed against a target antigen, could be applied to the analogous situation of detecting a lectin binding to carbohydrate structures using lectin histochemistry. With some minor technical modifications, approximately similar methodologies apply; the technical aspects of lectin histochemistry for both light and electron microscopy are reviewed by ***(20)***. One of the advantages of lectin histochemistry is that, like immunohistochemistry, it can be applied to routinely fixed and processed paraffin wax embedded tissue blocks, facilitating retrospective studies and studies of routinely archived surgical specimens. Significantly, the development of lectin histochemistry opened the way for far more specific, detailed and revealing mapping of carbohydrate structures in tissues than was previously possible using traditional histochemical techniques such as staining with periodic acid Schiff or Alcian blue.

While conditions of fixation and tissue processing for lectin histochemistry of cells and animal tissues may be specially adapted to optimise labelling results, lectin

histochemistry works well on routinely formalin-fixed and paraffin wax embedded specimens, such as those produced in routine cellular pathology hospital laboratories ***(20)***. This means that clinical studies associating carbohydrate-profiling of tissues with disease states or with disease progression can be very informative. Moreover, archival paraffin wax embedded tissues retain their carbohydrate profiles, facilitating powerful retrospective studies with long patient follow up. Examples of such studies are featured in section 3.2. These types of studies have advanced our understanding of the biological significance, and therefore function, of carbohydrate-receptor interactions, and of altered cellular glycosylation, in health and disease.

2.2 A wider range of tools become available

As far as lectin histochemistry is concerned, today, purified lectins from diverse sources are available for use in research. The majority are from plant sources, mostly derived from seeds (eg *Phaseolus vulgaris*, kidney bean; *Dolichos biflorus*, horsegram), but also bulbs (eg *Galanthus nivalis*, snowdrop), tubers (eg *Solanum tuberosum*, potato), fruit (eg *Lycopersicon escullentum*, tomato) and bark (eg *Sambucus nigra*, elderberry). Lectins from fungi (eg *Aleuria aurantia*, mushroom), invertebrates (eg *Helix pomatia*, Roman snail; *Limulus polyphemus*, horseshoe crab), fish (*Anguilla anguilla*, eel), snakes (eg *Naja mocambique moquambique*, Mozambique cobra) and marine algae (eg *Codium fragile*, green marine algae) also feature. Lectins are supplied in their native form and conjugated to fluorescent labels such as fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC), enzyme labels such as horseradish peroxidase and alkaline

phosphatase, biotin, digoxigenin and electron dense labels such as ferritin and colloidal gold. In recent years, there has been an expansion in the availability of lectins conjugated to modern fluorophores, such as those of the 'Dylight' and 'Alexa Fluor' ranges, which facilitate multiple labelling and are especially suitable for confocal laser microscopy and flow cytometry. A brief review of the main commercial suppliers of lectins for histochemistry reveals that around 100 different purified lectins are readily available to researchers.

Traditional lectin histochemical approaches elegantly reveal the distribution of specific glycoconjugates on the surface of cells or within tissues and can be used to map changes with development and in health and disease. As noted above, such lectin histochemistry commonly employs lectins derived from plants and invertebrates, but their selective binding to carbohydrate partners in animal or human tissues poses the question of what endogenous binding receptors (lectins) they are designed to complement, and what cellular responses are elicited *in vivo* by their interaction with such partners. One approach to identifying and isolating such endogenous tissue lectins is through the application of 'neoglycoproteins' or 'neoglycoconjugates'. Briefly, specific glycans are linked to a protein or synthetic polymer scaffold and their binding to cell- or tissue-bound lectins detected through techniques analagous to lectin or immunohistochemistry, termed 'glycohistochemistry' (see ***(21)*** for review). For example, Gabius et al (1990) ***(22)*** synthesised a neoglycoprotein based on the T antigen (also known as TF or Thompsen-Friedenreich antigen, it is a common cancer-associated antigen, β-Gal(1→3)GalNAc), see also section 3.2) and labelled cancer cell lines and tissue

sections of breast cancer to seek endogenous complementary lectin partners. Xu et al (2000) ***(23)*** explored changes associated with malignancy and tumour progression in testicular cancers using lectin histochemistry, glycohistochemistry and also immunohistochemistry to detect galectin-1, revealing changing patterns of glycosylation and endogenous lectins between primary tumours and metastases. Delorge et al (2000) ***(24)*** explored expression of galectin-3 and T antigen and their receptors in human head and neck squamous cell carcinomas, using immunocytochemistry and glycohistochemistry, revealing a relationship with de-differentiation during tumour progression.

There is clearly potential also, once endogenous lectins are sequenced, to produce recombinant human lectins which themselves could be employed in more specific, selective and targeted lectin histochmemical studies. A good example of this is described by Habermann et al (2011) ***(25)*** who labelled *zona pellucida* of bovine eggs for binding of recombinant galectins. However, this type of approach has not been widely adopted.

2.3 Lectin binding carbohydrate specificity

The carbohydrate-binding specificity of a lectin is most usually expressed in terms of the simple monosaccharide, or sometimes di- or tri-saccharide, that has been demonstrated to most effectively inhibit its binding. For example, *Dolichos biflorus* (horsegram) lectin is described as being alpha-GalNAc-binding or alpha-GalNAc-specific; *Arachis hypogaea* (peanut) lectin is said to be specific for β-Gal(1→3)GalNAc, T antigen. However, it is important to appreciate that a lectin is

usually recognising a structure that is larger and more complex than a single monosaccharide or disaccharide, and in three dimensions, taking into account specific glycosidic linkages, and lectin-receptor binding may involve potentially hydrophobic and electrostatic interactions ***(20)***. Thus, two lectins with apparently identical simple carbohydrate-binding specificity may actually recognise quite distinct profiles when used as tools in lectin histochemistry. A good example is Concanavalin A (Con A, the lectin from *Canavalia ensiformis*, jack bean) and *Lens culinaris* (lentil) lectins, both of which are generally defined as being Man- and Fuc-binding, and, indeed their binding can be effectively inhibited in the presence of free Man or Fuc. However, their binding patterns in histochemical studies are quite distinct ***(20)***. We have reported distinct tissue-labelling patterns achieved using a panel of 26 different lectins all reported to be specific for GalNAc ***(26)***.

An added layer of complexity is that for the majority of commercially available lectins used in histochemistry studies, the actual binding partner(s) present in tissues, or indeed their natural carbohydrate binding partners in the organisms from which they were isolated, is often poorly defined. The complexity involved in understanding the detailed carbohydrate-binding properties of lectins is exemplified by research into the characterisation of *Helix pomatia* lectin (HPA, from the Roman snail), which is of interest because histochemistry using this lectin identifies cancers that have metastasised and therefore have a poor prognosis (see section 3.2). Inhibition studies in the 1970's and 1980's first demonstrated that α-GalNAc is the best monosaccharide inhibitor of HPA binding, and that its binding can also be inhibited to a lesser extent by α-N-acetylglucosamine (α-GlcNAc) and also, although

poorly, by α–Gal. More recently, detailed studies have reported its binding preferences as: Forssman antigen (α-GalNAc1-3GalNAc-R) > Blood group A substance (α-GalNAc1-3[α-Fuc1-2]Gal) > Tn antigen (α-GalNAc-Ser/Thr) > α-GalNAc > α-GlcNAc ***(27)***. The preference of HPA for binding Forssman and Tn antigens is of interest because both have been described as cancer-associated antigens (see section 3.2 for more detail about Tn antigen). HPA has also been reported to recognise an incompletely characterised mono-sialylated oligosaccharide, termed HPAgly-1, that is present in aggressive breast cancer ***(28)***. The crystal structure of the lectin has been elucidated, demonstrating that it is a hexameric molecule with groupings of three GalNAc-binding sites at each end of the hexamer, and that the carbohydrate binding sites are created by a network of hydrogen bonds ***(29)***. Detailed analysis of the structural basis for recognition of Forssman and Tn antigens has been carried out ***(30)*** revealing some of the complexities of lectin-carbohydrate binding. These types of studies exemplify the over simplification of describing a lectin as, for example 'GalNAc-binding'.

Even when their precise binding-partners are not fully elucidated, lectins can still be used effectively to define the distribution of highly specific glycan structures in lectin histochemistry. For example, several lectins can be used to map the distribution of specific sialylated glycans. Sialic acid (SA) is a common terminal monosaccharide in glycan chains. *Limax flavus* lectin will recognise terminal SA residues on N- and O-linked glycans, regardless of linkage, which are commonly attached to a sub-terminal Gal residue ***(31)***, and is therefore useful to map the distribution of sialylated glycans overall. *Maakia amurensis* lectin preferentially recognises α-2,3 linked SA. *Sambucus*

nigra and *Polyporus squamosus* lectins both preferentially recognise α-2,6 linked SA, but *Polyporus squamosus* preferentially recognises SA terminating the chains of N-linked glycans, while *Sambucus nigra* lectin not only recognises SA terminating O-linked glycans chains, but also has some preference for binding terminal GalNAc, giving it a much broader binding specificity ***(32-34)***.

As more lectins are isolated and made available commercially, and as the fine carbohydrate-binding preferences of lectins are better defined, thus the capacity for lectin histochemistry to reveal fine detail of cellular of glycosylation will increase, especially when combined with cutting edge microscopy techniques. For example, Walski et al (2017) ***(35)*** mapped the spatial distribution of glycans on midgut cells from larvae of a common agricultural pest, the cotton leafworm *Spodoptera littoralis*. They use a panel of seven fluorescently labelled lectins and imaged the results by confocal microscopy giving stunning detail. Harrison et al (2018) ***(36)*** used fluorescently labelled *Lycopersicon esculentum* (tomato) lectin to label the brain vasculature of Alzheimer's disease patients and, after tissue clearing, imaged using light sheet and confocal microscopy and reconstructed the tissue in three dimensions. Moreover, the increasing availability of high-throughput screening platforms, such as lectin microarrays (described is section 4.4), are making detailed glycan profiling of samples much more accessible.

3 Major applications of lectin histochemistry

3.1 Mapping glycosylation changes in normal human and animal tissues.

The facility of lectin histochemistry to reveal glycosylation profiles of cells and tissues has resulted in a substantial literature of papers describing histochemical studies of various normal animal and human tissues. Some describe lectin binding characteristics of a particular tissue or tissues in selected species and compare results to what has been described for the same tissues in other species. Others, describe changes in lectin binding during embryogenesis or with tissue differentiation. A profile of such studies from the 1990's when these types of studies were most prolific, for example, is summarised in ***(37)***. The majority of studies have been simple descriptive ones and such investigations have generally not been carried out in any systematic way or in response to a well-defined research question. Thus, there remains much that lectin histochemistry can reveal regarding the glycosylation profiles of normal healthy tissues and, as described in section 3.2, there is currently much interest in exploring the 'glycomes' - that is, the profile of glycoconjugates synthesised by cells or tissues - of species, including humans, in development, health and disease.

3.2 Glycosylation changes associated with progression of malignancy revealed by lectin histochemistry

One area of lectin histochemical research that has proved especially fruitful has been the exploration of changes in cellular glycosylation associated with transformation of normal cells to malignant, and changes associated with cancer progression. Interest began with early cell agglutination studies, such as those described in section 1.2. Aub et al (1963) ***(38)*** were amongst the first to investigate the idea that one of the differences between normal and malignant cells was related to aspects of the cell

surface. They incubated malignant and normal cells with a range of enzyme preparations and noted that a crude preparation of lipase from wheatgerm agglutinated malignant cells and left normal cells unaffected. Later work proved that it was not the lipase that was responsible for the selective cell clumping, but was the action of a lectin, now referred to as wheat germ agglutinin (WGA, from *Triticum vulgaris*), and much interest in WGA as a marker of malignancy resulted. Subsequent studies, such as ***(39,40)*** also reported that other lectins, such as Con A and soyabean agglutinin (SBA, from *Glycine max*) also could preferentially bind to, and agglutinate, cancer cells (for example, see ***(41)*** for review).

With increasing popularity of lectin histochemistry, and, as indicated in section 2.1, its effective application to routinely fixed and processed archival clinical specimens, many studies in the 1980's and 1990's, in particular, investigated the ability of lectins to distinguish normal from malignant cells, or stratify tumours into different histological types or with different clinical outcomes. As a result, it is now well established that fundamental changes in cellular glycosylation are associated with cancer, and that some hold prognostic and functional significance. Some commonly described glycosylation changes associated with cancer are reviewed in ***(42)***; of special note, as far as lectin histochemistry is concerned, are truncation of O-linked glycans, and an increase in β-1,6 branching of N-glycans (fuller descriptions of glycosylation mechanisms are given in ***(42-44)***).

Failure in normal carbohydrate chain extension results in truncation of glycan chains, and this has emerged as a common feature of many cancer types. Mucin type O-

linked glycosylation of proteins begins with the attachment of a GalNAc monosaccharide to a serine (Ser) or threonine (Thr) residue of the polypeptide. The resulting structure, GalNAc-α-O-Ser/Thr, Tn antigen, is then always extended in normal adult human cells to form a range of basic core structures, or is capped by SA to yield sialyl-Tn which terminates chain extension. Interestingly, Tn antigen, which is detected by binding of the lectin HPA (the lectin has a nominal binding preference for GalNAc and will recognise glycans with terminal GalNAc including, but not exclusively, Tn antigen ***(45)***) and *Vicia villosa* (VVA, hairy vetch) isolectin B4, is frequently unelaborated in cancers, and cancers exhibiting high levels of Tn antigen are often more aggressive. For example, there have been numerous studies showing that HPA binding is a powerful marker of poor prognosis in many cancer types, most notably breast and colorectal cancer (for review see ***(42,46)***). As Tn antigen is normally cryptic, its exposure on cancer cells results in an immune response in cancer patients and it has been explored as a potential target for immunotherapy ***(47,48)***. β-Gal(1→3)GalNAc or T antigen is formed by the attachment of a β–1,3 linked Gal to Tn antigen. It can be detected by binding of peanut agglutinin (PNA, from *Arachis hypogaea*), has also been described to be associated with poor prognosis and aggressive biological behaviour in colorectal and other cancers (eg see ***(49)*** for review).

β-1,6 branched N-linked glycans can be detected using histochemistry for the binding of *Phaseolus vulgaris* (common bean) leukoagglutinin (PHA-L) and an increase in this type of glycosylation is associated with aggressive biological behaviour and poor prognosis in cancers, including breast and colorectal cancer

(50,51). The reason for the glycosylation change is increased activity of the glycosyltransferase that underlies the synthesis of these structures, GlcNAc transferase V *(52)*. The association between the transferase activity, the synthesis of β–1,6 branched N-linked glycans and metastatic potential is confirmed in animal models of metastasis (reviewed in *(42)*). Moreover, the mechanisms by which this change influences cancer behaviour have been elucidated: the altered glycosylation of matriptase, an enzyme that is key to activating a cascade of protease activity involved in cancer cell invasion, renders it more resistant to degradation, thereby upregulating the entire cascade. Swainsonine, an inhibitor of one of the early stages in the synthesis of N-linked glysosylation, has been tested in clinical trials as a potential anti-tumour therapy, although it was not well tolerated by patients *(53)*.

Thus, as exemplified by detection of β–1,6 branching N-linked glycans by PHA-L and detection of the normally cryptic Tn antigen by HPA, described above, lectin histochemistry to detect alterations in glycosylation in diseases such as cancer may have potential in identifying further novel targets for early detection, screening, and innovative treatment approaches. In recent years there has been much interest in extracellular vesicles (EVs), nano-sized membrane-bound 'packages' that are released by all cells and carry 'cargo' (of proteins, lipids nucleic acids and other substances) that is reflective of the parental cell. There is evidence to suggest that EVs released by cancer cells exhibit altered glycosylation and that they can induce functional effects in other cells (reviewed by *(54)*). There is currently interest in the idea that EVs released by cancer cells and present in body fluids such as blood or urine may have potential as carrying biomarkers for diseases, including cancer. As an

example, Yamamoto et al (2019) ***(55)*** isolated tumour-associated EVs from melanoma, glioblastoma, lung and colon cancer cells using a lectin from *Oscillatoria agardhii* (blue green algae; it recognises high mannose structures) immobilised on beads.

4 The next generation of lectin-based technologies

4.1 Lectins to decipher the complexities of the glycome

The chemistry and biology of glycosylation are topics that lie outside the scope of this chapter and are well described by ***(43,44)*****.** The chemistry of carbohydrates is such that the 'sugar code' or glycome, comprises glycan structures with a degree of diversity several orders of magnitude higher than that achieved by proteins or nucleic acids (see ***(56,57)*** for review). Glycoconjugates carrying this rich code are positioned on eukaryotic cell surfaces and are ideally placed to function in diverse biological mechanisms including cell identity, recognition, signalling, growth regulation, adhesion and in development, health and disease in all living things, and their significance is only just beginning to be appreciated and explored. This is a topic that is at the forefront of biology, but which has lagged behind exploration of proteins and nucleic acids because the tools to enable exploration are only just really becoming available. In many ways, lectin histochemistry, whilst a long-established and commonly employed technique to map glycosylation in cells and tissues, has yet to reveal its full potential because the carbohydrate binding-partners recognised by lectins are often not fully characterised and their biological functions are still poorly understood. However, exciting developments indicate that this may soon change

and there is increasing interest in exploring the glycome and its functional significance.

4.2 Lectin histochemistry complements other methods of exploration

While the literature reveals that simple lectin histochemical mapping studies of tissues remain popular and informative, increasingly studies are being designed in which lectin histochemistry is just one of a range of techniques employed to address a specific and carefully formulated research question regarding the functionality of the glycome.

A good example of this is the investigation of the functional significance of altered O-linked glycosylation in cancer, described in section 3.2. Simple lectin histochemical studies in the late 1980's and 1990's first established that increased binding of HPA preferentially recognises glycans synthesised by cancers that are more biologically aggressive, and it is therefore a useful prognostic marker (see ***(42,45)*** for review). However, the precise identity of the binding partner(s) recognised by HPA in cancers has still not been completely established, nor have their functional significance in mechanisms of cancer progression been completely elucidated. Initially, lectin histochemistry was combined with lectin affinity chromatography to isolate HPA-binding glycoproteins isolated from cancer and normal cells and tissues and characterisation was attempted using SDS-PAGE and western blotting ***(45,58)***. This revealed that the lectin was recognising a heterogeneous range of proteins bearing GalNAc-moieties, including, but not exclusively the Tn antigen, GalNAc-α-Ser/Thr.

Studies have also been conducted where the HPA-binding glycans have been cleaved from lectin affinity-purified glycoproteins and analysed using a range of chromatography approaches ***(49,59)***. Peiris et al (2015) ***(60)*** employed an elegant glycoproteomic approach to identify specific HPA-binding glycoproteins that were increased in colorectal cancer specimens. Here, studying human cancer samples where clinicopathological features of the patients were known, a combination of traditional lectin histochemistry was combined with HPA affinity chromatography to isolate proteins of interest, then their analysis by SDS-PAGE and western blotting, two dimensional electrophoresis (2-DE) and identification by matrix assisted laser desorption ionisation mass spectroscopy. Moreover, prediction of potential glycosylation sites on the proteins was performed using an *in silico* bioinformatics approach, and correlations were also made with the status of cancer-related genes p53, using immunohistochemistry, and KRAS, using polymerase chain reaction (PCR). Meanwhile, Bapu et al (2016) ***(61)*** demonstrated that the truncated HPA-binding GalNAc-glycans were involved in the adhesion of cancer cells to endothelial monolayers *in vitro*, suggesting that they may functionally involved in cancer cell adhesion to the lining of blood vessels during hematogenous dissemination of the disease. Such studies illustrate the potential for using lectin histochemistry, alongside other techniques, to answer a defined question related to glycosylation, to more clearly define biomarkers as novel targets for disease treatment or to more fully understand disease processes at a molecular level.

4.3 Recombinant and engineered lectins

While, as indicated in section 2.3 the precise binding partners of many lectins remain incompletely defined, still progress is being made to elucidate their properties in more detail. Moreover, advances in molecular biology mean that the concept of engineering lectins with clearly defined, altered or improved carbohydrate-binding specificity is becoming an exciting reality, potentially circumventing the problems of batch to batch variation in the activity and properties, weak binding affinity, and the often broad and incompletely defined binding repertoire of the naturally occurring product. Potentially, lectins can be engineered that bind to less common glycan structures where no naturally occurring lectin-partner is known. Such recombinant lectins are commonly expressed in bacterial or yeast. This topic is reviewed by ***(57)***. Many of the advances in this field have come from the increasing popularity, and utility of lectin microarrays (section 4.4) to explore glycosylation of complex samples, especially for human disease biomarker discovery. In this arena there has also been interest in expanding the repertoire of recombinant mammalian lectins available, since exploring the human glycome using lectins derived from, for example, plants and invertebrates, is unlikely to address the specific complexities of human glycosylation.

One good example, is that of HPA which, as described in sections 3.2 and 4.2 can be used to identify cancers that have metastasised. Markiv et al (2001) ***(62)*** describe the various studies from the 1960's onwards aimed at solving the structure and elucidating the binding site of the lectin. They finally isolated two closely related and GalNAc-binding isolectins from *Helix pomatia* and expressed recombinant forms of the lectins that had a slightly higher affinity for GalNAc than the native lectins. They

propose that the recombinant form, being more homogeneous and well characterised, might provide a more robust tool for cancer prognostication than the naturally variable native product.

There are also more elaborate and complex attempts to engineer lectins for enhanced properties. Maenuma et al. (2008) ***(63)*** introduced mutations into the carbohydrate binding domain of the sialic acid binding lectin from *Maackia amurensis* (the Amur maackia or Chinese yellow wood) producing thirty-five different lectin variants with distinct carbohydrate-binding properties which they used to distinguish the cell lineages of carcinoma, myeloid, fibroblastic and melanoma cells. Yabe et al (2007; 2009) ***(64,65)*** used a strategy of introducing random mutations through error-prone polymerise chain reaction to alter the carbohydrate binding preference of a the Gal-binding domain of the lectin ricin B-chain to α2-6 linked sialic acid, then further reengineered the lectin to have a preference for branched N-glycans carrying multiple α2-6 linked sialic acid residues, a feature not seen in naturally occurring α2-6 sialic acid-binding lectins from *Sambucus nigra* (SNA, elderberry) and *Sambucus sieboldiana* (SSA, Japanese red elder), and no agglutinating activity.

4.4 Lectin microarrays to explore the glycome and search for glycosylation-associated disease biomarkers

This complexity of glycosylation (reviewed by ***(43,44)***), makes isolation of individual glycan structures from a complex mixture, or glycoconjugates exhibiting a specific glycosylation profile for study, technically difficult. Moreover, analysis of complex

carbohydrates has been hampered for many years by the technical challenges imposed by the unique chemistry of this group of molecules, and detailed analysis of these complex structures remains a specialist and complex field (see ***(57,66,67)*** for review). Lectin interaction with a potential carbohydrate binding partner can thus only be examined in the presence of heterogeneous, similar, but subtly different glycoforms (reviewed by ***(68)***). The study of such interactions is further complicated because lectin-carbohydrate binding interactions are weak, and lectins are usually multivalent, thus enhancing binding through co-operation where binding characteristics at one site are influenced by the binding status of others. One approach to overcoming these issues, as described in ***(68)***, is through computer simulation. Alternatively, there have in recent years been great advances in development – and use - of high throughput screening platforms.

Lectin-carbohydrate binding interactions have been studied using several different microarray approaches. For example, libraries of carbohydrate structures immobilised as microarrays can be used to detect the presence of endogenous lectins (for example, ***(69,70)***). Alternatively, microarrays of immobilised antibodies directed against a panel of proteins can be allowed to bind to glycoconjugates in a complex sample, and then overlaid by labelled lectins in order to analyse the glyco-profiles present (for example ***(71)***). Lectin microarrays can also be used to capture glycosylated molecules from a complex sample and yield information about comparative glycosylation profiles (for example, ***(72)***). Each of these approaches facilitates the rapid screening of multiple glycan-lectin interactions. They have the

additional advantages that they do not require purification of sample or cleavage of the glycan from the protein, and allow rapid and sensitive 'glycomic' profiling. Lectin microarrays have, for example, been used for profiling global changes in mammalian *(73,74)* and bacterial *(75)* cell surface glycomes. However, these approaches do not allow detailed glycan analysis, and are instead suited to comparison of the glycoprofile of different samples.

A variety of different detection methods can be used in relation to microarrays. At their simplest, fluorescent, chemiluminescent or radioactive labels can be used in direct or indirect labelling approaches analogous to those employed in traditional lectin histochemistry. For example, glycoconjugates from biofluids or cell/tissue extracts may be labelled using a fluorescent tag such as cyanine-3 (Cy-3) or Cy-5 for direct detection before being loaded onto the array. Alternatively, lectin-glycoconjugate interactions can be detected using a biotinylated antibody directed against the glycoconjugate sandwiched with streptavidin conjugated to either horseradish peroxidase or, in a more sensitive assay, to a fluorophore and detected using confocal microscopy. Generally, such approaches are only able to detect lectin binding of the most abundant glycoconjugates in a sample, but modifications have been developed to improve sensitivity, including tyramide signal amplification *(76)*, enrichment of low abundance target glycoconjugates prior to their introduction onto the array, and detection using a highly sensitive evanescent-field fluorescence scanner *(72)*. In some instances, the binding capacity of the lectins can be enhanced by their orientation on the array, as exemplified by *(77)*. Wang et al (2014) *(78)*

developed a bead-based array where lectins were coupled to fluorescently-labelled microbeads, allowing glycan-lectin recognition to be performed three dimensionally.

Since carbohydrate recognition by lectins plays a role in such a diversity of biological processes, there is interest to more fully understand the nature of their binding interaction. Highly sophisticated approaches have been developed to study single molecule lectin-carbohydrate interactions. For example, Zhang and Yadavalli (2009) ***(79)*** described how a carbohydrate or lectin array could be fabricated based on a self-assembling monolayer and lectin-carbohydrate interactions interrogated at the single molecule level using atomic force microscopy. They imaged and measured interactions between Con A and mannosyl groups; specificity of binding was confirmed by inhibition of binding by free Man, and investigations at different pH values facilitated exploration of multivalent interactions. Atomic force microscopy has been employed in other studies to investigate carbohydrate-lectin interactions. For example, in ***(80)*** to study interactions of glycophorin A, immobilised on a mica surface or presented in the erythrocyte membrane, with *Psathyrella velutina* (the weeping widow mushroom) lectin, and in ***(81)*** to study interaction of Con A with carbohydrate microarrays.

Lectin microarrays represent a powerful tool for glycoproteomics, that is, exploring the repertoire of glycans on proteins expressed by cells or tissues, focussing on changes in glycosylation during normal biological functioning, and in disease. They can be used for analysis or comparison of complex biological samples, such as biofluids or cell or tissue extracts. This approach holds particular potential for high

throughput screening for the altered protein glycosylation that has been reported to accompany development of malignancy and other diseases, thus holding promise for biomarker discovery, and potentially the development of early diagnostics or prognostic markers (reviewed by ***(82,83)***). Most lectin microarrays are composed of lectins derived from natural sources, but arrays featuring recombinant lectins have also been developed ***(84)***. In more complex studies, the glycoconjugates bound to lectin microarrays have been analysed for mass using mass spectrometry (for example ***(85)***) or their kinetics, affinity and specificity have been explored using surface plasmon resonance (SPR) (for example, ***(86)***).

Most lectin microarray studies have focused on screening biofluids, especially plasma or serum, for potential biomarker discovery and one limitation is that samples are likely to contain a very heterogeneous range of glycosylated molecules, only a tiny proportion of which are derived from the diseased tissue, for example tumour, itself. Thus, glycoconjugates of interest may first need to be purified or concentrated from the dilute sample, and results often need to be considered alongside traditional lectin histochemistry or other approaches. As an example, Jiang et al (2015) ***(87)*** first concentrated a candidate serum biomarker GP73, that had been reported to be up-regulated and differently fucosylated in individuals with liver disease, using immunoprecipitation before applying it to a 50-lectin antibody overlay microarray. This approach identified a panel of five lectins that appeared to distinguish between samples taken from patients with cirrhosis versus liver cancer. Results of the microarray were supported by analysis of serum samples by western blotting and ELISA. An example of lectin microarrays being used alongside traditional

lectin histochemistry is a study by Huang et al (2014) ***(88)*** who, using a 37-lectin microarray, were able to distinguish the glycosylation profiles of proteins extracted from frozen specimens of gastric cancer versus gastric ulcer. Results were confirmed using traditional lectin histochemistry on formalin fixed paraffin wax embedded sections of tissue. Two lectins, from *Maclura pomifera* (MPL, Osage orange) and *Vicia villosa* (VVA) were identified as biomarkers for gastric cancer. Another example is that of Nakajima et al (2015) ***(89)*** who analysed the glycan profiles of proteins extracted from formalin fixed, paraffin wax embedded archival specimens of 53 stage I-III colon cancer using a 45-lectin microarray. Again, the microarray was employed alongside traditional lectin histochemistry. They identified that *Agaricus bisporus* (ABA, common mushroom) binding was associated with subsequent recurrence of disease in these patients. Both of these studies illustrate the utility of lectin arrays to interrogate samples extracted from routine clinical tissue samples, opening the potential for large scale screening for disease biomarkers.

5 Conclusion

Lectins first elicited the interest of researchers more than one hundred years ago owing to their curious ability to agglutinate living cells. When it became apparent that they could do so through their selective recognition of cell-surface carbohydrates, and furthermore that different types of cells exhibited different patterns of glycosylation and, thus, different lectin-binding profiles, they became, and remain, the tool of choice for exploring the glycome. Lectin histochemistry has been instrumental in mapping cellular glycosylation in different species, different cell

types and in normal and disease processes. In recent years, the remarkable potential of the 'sugar code', its complexity, and its as yet incompletely explored functional importance in all aspects of biology has given new impetus to the development of tools to decipher it. Traditional lectin histochemistry still has a place, alongside developing technologies, in revealing fundamental aspects of glycosylation, while new developments hold promise of a new generation of lectin-based research approaches.

6 References

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