Glycoproteomics characterization of immunoglobulins in health and disease

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Chapter 6:

Discussion

Table of Contents

6.1: General thoughts on glycosylation

At a first glance, glycan synthesis in humans may seem to be an energetically costly and needlessly elaborate system. The glycosylation machinery takes up an estimated 1% of genes in humans (1), and many of the enzymes involved in glycosylation have similar tasks, the most egregious offender being the GalNAc-transferase, of which approximately 20 isoforms exist in humans (2). Furthermore, during *N*-glycan synthesis various building blocks are added which are removed in subsequent steps. One may well ask whether this level of complexity is necessary, especially since some of the structural alterations in proteins mediated by glycosylation could likely be achieved by other means as well. This is evidenced by the fact that the rat homologue of a human glycoprotein was found not to be glycosylated, but instead exhibited similar properties (i.e. receptor binding) through changes in the protein sequence (3), and similarly, glycosylation sites are not always conserved in related proteins (4). On the other hand, it cannot be stated that there is a high level of redundancy, as the loss of a single enzyme, even among the GalNAc-transferases, can result in serious developmental disorders (2, 5).

Glycosylation can thus be seen as a useful process which, due to its multifaceted nature, has been integrated into many different pathways over the course of evolution. One advantage of glycosylation in contrast to mutations in single protein genes, is the fact that mutations in genes involved in the glycosylation machinery can affect a large number of proteins simultaneously. In simple, single-celled eukaryotes such as yeast, *N*-glycans vary only in the number and branching of mannoses. As multicellular organisms emerged and grew continuously more complex, glycosylation was embedded into new pathways, such as cellular adhesion, signalling and degradation of asialylated glycoproteins. Simultaneously, glycosylation became more diverse, with a larger number of glycan monosaccharides, linkages and motifs, likely to accommodate the growing number of functions, although pathogen evasion has also been argued to play a role.

6.2: Analytical challenges

6.2.1: Partial occupancy of glycosylation

Chapter 3 describes the novel discovery of *O*-glycans in the hinge region of IgG3. The *O*-glycosylation sites were not fully occupied, as an *O*-glycan was only present at approximately 10% of the sites, but considering the total number of potential *O*-glycosylation sites, it is

possible that roughly half of all IgG3s carry at least one *O*-glycan. The fact that this structural feature evaded notice for over seventy years since the IgG protein was discovered (6) and over half a century since the first description of its *N*-glycosylation (7), is a testament to the fact that novel glycosylation discoveries are not limited to less-characterized proteins.

Partial site-occupancy of post-translational modifications (PTMs), including glycosylation, could be present on many proteins that have been sequenced in the past decades. If a modification is only present on a fraction of the protein molecules, the non-modified peptide will still be identified using a bottom-up proteomics approach. Hence, without specific attention to a modification, it can easily be missed using the automatic search routines. Therefore, in order to discover modifications, such as glycosylation, one must actively look for them, and for many PTMs this option is already available in commonly used software, e.g. MASCOT (8). However, for glycosylation this is not as straightforward due to the large number of possible glycan structures. Nonetheless, several software programs capable of analyzing glycopeptide fragmentation data are available (9-11). Alternatively, the chemical cleavage and labelling of glycosylation sites before their analysis may circumvent the problem of glycan structure heterogeneity (12-14). Another approach bypasses structural diversity by producing *O*-glycoproteins in a cellular system modified to generate only truncated *O*-glycans (SimpleCell) (15, 16), but unfortunately this method encompasses a lectin-purification step, thus eliminating the possibility of assessing the occupancy of *O*-glycosylation sites.

Potential *N*-glycosylation sites are easily identified due to their consensus sequence, which forms an easy target for investigation. Partial occupation of *N*-glycosylation sites occasionally goes unnoticed, as evidenced by the recent finding of a second *N*-glycan in IgG3 (17), but in general, for well-characterized proteins, sites of *N*-glycosylation are known, even if the exact range of glycan structures is often unexplored (18).

O-glycans are much more likely to go unnoticed, for various reasons. Firstly, *N*-glycans can easily be released using the enzyme *N*-glycosidase F, but for *O*-glycans chemical methods are required, which do not work as well. Secondly, the lack of a single sequence-specific *O*-GalNActransferase, and therefore lack of a consensus sequence for *O*-glycosylation, makes it difficult to predict where *O*-glycans are likely to show up (19). Hence, the prediction tools which are available are not yet very accurate. For example, two software tools deemed all

three IgG3 *O*-glycosylation sites described in chapter 3 'not *O*-glycosylated', based on the IgG3 protein sequence, while assigning the status '*O*-glycosylated' to 17 or 37 other amino acid residues in IgG3 (GPP (20) and YinOYang1.2 (21), respectively). A third tool assigned a 50% chance of *O*-glycosylation to the three identified *O*-glycosylation sites, but also assigned a higher chance to 13 other threonine and serine residues within IgG3 (NetOGlyc 4.0 (16)).

A third reason that *O*-glycosylation analysis is challenging is the heterogeneity in occupation of *O*-glycosylation sites (macroheterogeneity). Potential *O*-glycosylation sites often occur in clusters, with some sites occupied and others not, and the pattern of occupied sites can be influenced by environmental factors (13). The hinge regions of IgA1 and IgD are good examples of this: IgA1 isomers with differentially occupied *O*-glycosylation sites have been described to occur naturally in human plasma (22). Different *O*-glycosylation sites have been described for IgD, indicating that *O*-glycosylation isomers could also be present there (23, 24). While it is difficult to determine due to the sparseness of studies on glycosylation occupancy in general and *O*-glycosylation specifically, we expect that partial occupancy is more widespread among *O*-glycosylation sites than *N*-glycosylation sites.

6.2.2: Cell-bound immunoglobulins

Another aspect of Ig glycosylation which has not yet been explored is cell-bound Ig. Glycosylation analysis of Igs has up to now been focused solely on soluble Igs, since these are easily extracted from body fluids. However, immunoglobulins are also present in cell surface-bound state as B cell receptors. B cell receptors originate from the same genes as the secreted Igs by way of differential mRNA splicing, with a transmembrane section at their C-terminus. Before B-cell maturation, all Igs in B cells are surface-bound, and upon antigen exposure the secreted variant is produced (25). It has not been studied whether these surface-bound Igs express glycosylation similar to that of soluble Igs. Many of the interactions that cell-bound Igs undergo are not shared with soluble Igs, such as the interaction with glycoproteins Ig-α (CD79A) and Ig-β (CD79B) to induce signalling reactions necessary for B cell survival and development, and whether these interactions are influenced by Ig glycosylation has not been investigated (25). Therefore, glycans which appear to have no discernible function on soluble Ig should be evaluated in the context of membrane-bound Ig as well.

Immunoglobulins can also be bound to the cell surface in a different way: by non-covalent binding to cell-bound receptors. Due to the high concentration of IgG in blood and the low binding affinity between FcγRs and non-antigen-bound IgG, the receptor-bound fraction of IgG is expected to be relatively small (26). However, *N*-glycosylation of Fc IgG has been shown to affect FcγR binding affinity, with the most influential factor being the core fucose: afucosylated glycoforms possess a 50-100x greater affinity for FcγRIIIa (27-29). While the level of afucosylated IgGs is on average less than 10% in blood (Chapter 5), one may speculate that the IgG fraction bound to FcγRIIIa on immune cells could show considerably lower fucosylation. Likewise, preferential binding of afucosylated IgGs to cellular Fcγ receptors may contribute to the depletion of these antibodies from the soluble pool.

In contrast to IgG, a substantial fraction of IgE is bound to FcεRI receptors on mast cells and basophils, due to both the exceptionally high affinity between IgE and FcεRI, as well as proximity between IgE-producing B cells and FcεRI-expressing cells (26, 30). It has not been evaluated whether the type of glycans attached to IgE influences binding to FcεRI, but any such preference would greatly influence the distribution of IgE glycoforms between the receptor-bound and free fraction. Furthermore, release of FcεRI-bound IgE could facilitate a higher yield of IgE from biological samples. The low concentration of IgE in plasma, at approximately 150-300 ng/ml (31-33), compared to 10 mg/ml for IgG in healthy individuals, is now often a limiting factor for analysis.

6.3: Functional aspects of Ig glycosylation

6.3.1: Immunoglobulin E

Since the publication of the IgE glycosylation analysis described in chapter 2, additional studies have been done on the functionality of the glycans on IgE. In their 2015 paper, Shade *et al.* convincingly show that the glycan at N394 (N275 in Uniprot) is essential for binding to the Fcε receptor I, and that the removal of this glycan – either enzymatically using the high mannose-specific endoglycosidase F1 (Endo F1) or through targeted mutation of the glycosylation site – abolishes binding (34). In addition, a second study used streptococcal endoglycosidase S (Endo S) to cleave the glycan at N394, and also showed that this inhibited receptor binding and mast cell activation (35).

Furthermore, Shade *et al.* adopted the site-specific IgE glycopeptide analysis method described in chapter 2 to characterize the glycosylation of their recombinant IgE samples. They managed to improve on the method, since they only reported the use of two enzymes – trypsin and chymotrypsin – to observe the glycosylation at each of the six IgE glycosylation sites, while the original method required a third enzyme – proteinase K – to observe the glycosylation at N265 (N146 in Uniprot). Unfortunately, Shade *et al.* did not list the glycopeptide sequences or *m/z* values which they observed, which makes it difficult to compare both methods. They reported using trypsin to observe the glycopeptides at N265, which, for the standard IgE sequence as listed by Uniprot (36), would result in a peptide mass of over 7 kDa. Considering that most of the N265 glycopeptide masses would then be above 9 kDa and our measuring range only went up to *m/z* 1800, which would allow only observation of 6+ or higher charged ion species, it is not surprising that we did not observe this glycopeptide.

Shade *et al.* further report high mannose glycosylation at N394, while the other IgE sites show mostly complex-type glycosylation, which is in agreement with our results as described in chapter 2. However, they find a high degree of tetra-antennary glycans (over 50% in two of the IgE sites), as well as LacdiNAc structures and antennary fucosylation, which we did not observe in IgE derived from healthy individuals. This can be attributed to the fact that their IgE was produced in HEK cells, which can result in different glycosylation (37, 38). Probably for the same reason, Danzer *et al.* report both high mannose and complex *N*-glycans at N394, while we found solely high mannose *N*-glycans at this site (35). Moreover, the HEK cell origin of the IgE could have been the reason why Shade *et al.* encounter glycosylation at N383 (N264 Uniprot), while in chapter 2 we report that this site was unoccupied on our IgE samples. This is supported by another recent study, which confirmed that N383 was unoccupied in patient-derived IgE (39). To have a clearer picture of the influence of IgE glycosylation on FcεRI binding, experiments with polyclonal IgE samples are warranted.

A subject of further study should be whether the type of high mannose glycans (i.e. the number of mannoses) at N394 can modulate the interaction between IgE and FcεRI. To this end, a setup similar to that of Shade *et al.* could be utilized, with pre-treatment of IgE with mannosidases or mannosyltransferases to generate high mannose glycans with a varying number of mannoses. Of note, addition of mannose residues with mannosyltransferases has the drawback that this could generate structures with over nine mannoses, which are not

normally present in humans. Cleavage of mannoses, with mannosidases carefully chosen to match the different mannose linkages present in high mannose glycans, therefore appears a more appropriate strategy.

6.3.2: Immunoglobulin G

Homologous to N394 on IgE is the glycosylation site N297 on IgG, which is occupied by diantennary complex-type *N*-glycans and has been implicated in the effector functions of IgG. Despite a significant number of studies devoted to the effect of IgG glycosylation on immune reactions using various types of assays, the outcomes are often inconsistent and the exact role of IgG Fc glycosylation and its pathogenicity in autoimmune disorders remains unclear.

In autoimmune patients, IgG is often known to exhibit a decreased level of galactosylation and sialylation and an increase in fucosylation (40, 41), as described for relapsed ANCA vasculitis patients in chapter 4. This is in line with the association between IgG agalactosylation and the inflammation marker CRP (chapter 5). Sialic acid attachment requires a galactose residue, and therefore sialylation is highly correlated with galactosylation and both show similar associations with disease or metabolic markers. However, after correction for galactosylation, sialylation did not appear to be associated with either autoimmune disorders (chapter 4) (42) or CRP levels (chapter 5).

In vivo studies in mice have shown that sialylated IgG has an anti-inflammatory effect in an arthritis mouse model; sialidase-treated IgG failed to induce such an effect, indicating that sialylation, and not galactosylation, is responsible (43). Several other studies also observed that sialylated IgG shows decreased FcγR binding and/or ADCC capacity (44, 45). However, other studies did not find a negative effect of IgG sialylation on FcγR binding or ADCC capacity (46, 47). In contrast, galactosylation is described to augment FcγR-mediated effector functions (46-50).

Complement adds another layer of complexity to the interaction between IgG glycosylation and effector functions. Both galactosylation and sialylation show a positive effect on the ability of IgG to activate the classical complement pathway according to C1q-binding and cell-based assays (47, 51). The lectin pathway, which is triggered by binding of IgG to mannose binding lectin (MBL), has been proposed to be involved in immune activation by

aglycosylated IgGs in autoimmune patients (52), but recent studies have not observed any involvement of MBL (47, 53). Furthermore, a recent paper by Karsten *et al.* describes a novel pathway by which IgG interacts with inhibitory FcγRIIB and Dectin-1, leading to suppression of C5a-dependent inflammatory reactions, but only if the IgG is highly galactosylated (sialylation played no role) (54). However, these reports clash with the study by Nimmerjahn *et al.*, which reported that complement did not play a role in the *in vivo* activity of either galactosylated or agalactosylated IgG (53).

To summarize, we have the following conundrums: according to several *in vivo* studies sialylation mediates anti-inflammatory reactions, but patient IgG profiles do not show a direct association of disease activity or inflammatory parameters with sialylation after correction for galactosylation. Furthermore, galactosylation is low in autoimmune patients, but high galactosylation appears to be a more effective immune activator according to *in vitro* ADCC studies. The exact way in which IgG glycosylation and inflammatory responses are linked is thus poorly understood.

6.4: IgG glycosylation as a biomarker

In chapter 4, we described the changes seen in ANCA vasculitis patients who relapsed, compared to patients who remained in remission: IgG of relapsers exhibited a significantly lower level of galactosylation and sialylation, as well as a non-significantly higher fucosylation and lower bisection. In chapter 5, we described that individuals with a high level of inflammation, as determined by high CRP and IL-6, also showed a significantly lower level of galactosylation and sialylation and a higher level of fucosylation. As discussed above, it is not known exactly how, or even if, the glycosylation of IgG can modulate immune reactions. However, similar glycosylation patterns have been seen in many auto-immune diseases and are thought to reflect a state of inflammation (40, 55). As such, the glycosylation of IgG could be utilized as a biomarker for autoimmune diseases or relapse thereof.

The complexity of IgG glycosylation analysis, with various glycosylation features derived from individual glycan measurements, may appear to be a disadvantage, but can also be seen as a strength. The multidimensionality of IgG glycosylation measurements could provide a more specific indication as to the ailment of a patient than the level of a single clinical

parameter such as CRP. The aforementioned combination of low galactosylation and sialylation and high fucosylation is seen in many autoimmune diseases, while low fucosylation appears to be associated with certain infections, as will be discussed below.

In chapter 5 we described that individuals with a cytomegalovirus (CMV) infection exhibit a significantly lower degree of IgG fucosylation within the LLS cohort: seropositive individuals exhibited 90.6% fucosylation, while 91.7% was seen in others. While these differences are relatively small, it should be noted that these are individuals with a latent, asymptomatic CMV-infection, and the amount of CMV-specific IgG as a proportion of the total IgG is therefore likely small. We also examined CMV-specific IgG of three unrelated samples, and this antigen-specific IgG subpopulation showed a much lower degree of fucosylation, at approximately 70% (unpublished data). Literature also reports several instances of antigen-specific IgG with low fucosylation: HIV-specific antibodies have been reported to exhibit approximately 75% fucosylation (56). Extremely low IgG fucosylation levels (as low as 12%) have also been observed for maternal IgGs against foetal red blood cells in haemolytic disease (57). In contrast, PR3 ANCA-specific IgGs exhibit significantly higher fucosylation compared to total IgG (chapter 4), similar to several other auto-immune conditions (40, 41). Thus, it appears that specific glycosylation changes can be induced in antigen-specific IgG, and that these vary depending on the nature of the inflammation. Autoantibodies show increased fucosylation, while antibodies against foreign compounds repeatedly appeared to carry afucosylated *N*-glycans, which in the case of the often predominant IgG1 subclass is considered to be more inflammatory with respect to induction of ADCC (27-29). However, the division is likely not as clear-cut as this, since no fucosylation difference was reported in total IgG in chronic hepatitis patients before and after treatment (58) or in antigen-specific IgG after influenza or tetanus vaccination (59).

A disadvantage of IgG glycosylation as biomarker is the large inter-individual variation. Part of this variation is age- and sex-related, and in fact this may reflect underlying differences in inflammation: older individuals are generally known to have a more inflammatory immune system, and also display lower galactosylation. Furthermore, post-menopausal women have the lowest degree of galactosylation (chapter 5), which fits with the higher abundance of auto-immune diseases in this group (60). The problem of large inter-individual variation could be circumvented by using IgG glycosylation as a personal biomarker for inflammation, for instance in individuals at risk for relapse, as we suggested in chapter 4.

In chapter 5 we did not find an association between IgG glycosylation and predisposition to longevity. This may be because a high level of immune activation can be either advantageous or detrimental, depending on the situation. Many elderly people die of opportunistic infections which their immune system is not able to handle. Furthermore, cancer occurs when an uncontrolled cell escapes the constraints of the immune system, and occurs more often in immunocompromised individuals (61). In both of these cases, it can be generally said that an active immune system is advantageous. On the other hand, inflammation is associated with and possibly contributes to heart disease, the leading cause of death in industrialized countries (62). Moreover, an overactive immune system can lead to auto-immune conditions, which, while they are often not fatal, can severely impact the quality of life. We expect that in individuals with a genetic predisposition for longevity, the immune system is finely balanced to avoid both under- and over-activity. For this reason, in future mortality analysis in the LLS study, it may be worthwhile to categorize mortality cases based on the cause of death, with two broad groups containing deaths related to over- and underactivity of the immune system.

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