Biologicals xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Therapeutic monoclonal antibody N-glycosylation – Structure, function and therapeutic potential

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ARTICLE INFO

Keywords:
IgG
Glycans
Glycosylation
Fc-receptors
ADCC
ADCP
CDC
Effector function
Therapeutic antibodies

ABSTRACT

Therapeutic antibodies (IgG-type) contain several post-translational modifications (PTMs) whereby introducing a large heterogeneity, both structural and functional, into this class of therapeutics. Of these modifications, glycosylation in the fragment crystallizable (Fc) region is the most heterogeneous PTM, which can affect the stability of the molecule and interactions with Fc-receptors *in vivo*. Hence, the glycoform distribution can affect the mode of action and have implications for bioactivity, safety and efficacy of the drug.

Main topics of the manuscript include: What factors influence the (Fc) glycan pattern in therapeutic antibodies and how can these glycans be characterized? How does structure of the Fc-glycan relate to function and what methods are available to characterize those functions? Although heterogeneous in their scope, the different sections are intended to combine current knowledge on structure-function correlations of IgG glycan structures with regard to Fc (effector) functions, as well as basic aspects and methodologies for their assessment.

1. Introduction

Monoclonal antibodies (mAbs) have been in use as therapeutics since the 1980s and became standard of care for a variety of diseases. The first mAb to be approved by the FDA was OKT3 in 1986 and thereafter additional molecules followed each year. The number of approved mAbs drugs continues to increase rapidly [1] and the clinical success is hence reflected in the continued commitment to the molecule type by the biopharmaceutical industry [2,3]. mAbs as therapeutic drugs offer advantages as compared to small molecules such as relatively high specificity, low toxicity and long half-life (i.e. weeks) in the human circulation. The key disadvantage compared to small molecules, in addition to the laborious production and purification processes involved, is the relatively high heterogeneity due to PTMs. These modifications however also offer opportunities to include additional functionalities and thereby provide possibilities to further fine-tune the efficacy of some therapeutic antibodies.

IgG-type antibodies are N-glycosylated at asparagine 297 (Eu-numbering, see Ref. [4]) on both of the two heavy chains in the Fc-portion. The composition of these glycans can significantly influence the therapeutic effect of the antibody due to effects on Fc-mediated interactions with different receptors [5]. This effect is observed for antibodies where the mechanism of action involves immune-mediated effector functions such as ADCC (antibody-dependent cellular cytotoxicity), ADCP (antibody dependent cellular phagocytosis) and CDC (complement dependent cytotoxicity). A correlation between FcγRIIIa phenotype and the clinical response has been reported, suggesting that ADCC activity could contribute to clinical efficacy [6–8]. As a result of this observed correlation, the concept of glycoengineering of therapeutic antibodies (addressed in detail in a subsequent section) was introduced and has thereby enabled improvements to the clinical efficacy of mAbs [9].

There are many examples of successful glycoengeneering, including i.e. the approved antibody therapeutics GAZYYVA and IVIG's. Glycoengineering by modifying host cells was used to significantly improve effector-functions for Obinutuzumab (GAZYVA, GAZYVARO) [10–13]. By modification of the glycan chains in the Fc-portion (resulting in higher levels of afucosylated glycoforms), the immune effector cell-mediated cytotoxicity of the target bound antibody could be significantly increased [14]. This example demonstrated the potential benefits of controlling the high heterogeneity of a PTM, which could otherwise be seen as a disadvantage compared to small molecule drugs.

The second well known example where the efficacy of clinically applied antibodies was shown to be influenced by the glycan pattern are intravenous IgG's (IVIG's; for a recent review see Ref. [15]). For a variety of autoimmune diseases, high dosages of IgG purified from human plasma are used to mediate anti-inflammatory activity. This effect could later be attributed to sialylated glycans in the Fc-portion

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https://doi.org/10.1016/j.biologicals.2017.11.001
Received 11 July 2017; Received in revised form 13 September 2017; Accepted 14 November 2017

and by specifically enriching antibodies containing this glycan, the efficacy of IVIG's could be increased [16–19]. Since the beneficial therapeutic effects of IVIG's are observed for many different diseases and due to the highly heterogeneous composition, multiple modes of action appear to exist and are still being elucidated. Glycan structures in the Fc-portion appear to play an important role in the many pathways used by IVIG's and the therapeutic potential can be predicted to be even greater with improved understanding of the mode of action [20].

In order to improve the understanding of N-linked glycans functionality, a brief introduction into antibody glycosylation is first provided, followed by a more technical introduction to glycoengineering, the analytical methods applicable to characterizing glycan structures and structural as well as functional consequences on antibody and antibody interactions with certain (Fc) receptors.

2. N-linked glycans in proteins

In eukaryotes, glycans can be co-translationally attached to asparagine residues of proteins in the endoplasmic reticulum. Oligosaccharyltransferase enzyme complexes recognize the sequence motif of asparagine-X-serine/threonine (N-X-S/T) in unfolded proteins, where X can be any amino acid with the exception of proline. Glycosylation efficiency appears to be affected by the nature of the adjacent amino acid [21]. In the case of IgG-type antibodies, such a motif is located in the CH2-domain of the heavy chains (see Fig. 1). A precursor glycan is initially attached by transferase-complexes and the presence of this glycan can then guide folding of the protein [22]. Proteins in the endoplasmic reticulum are then transferred via COPPII-coated transport vesicles out of the endoplasmic reticulum and into the different stacks of the Golgi apparatus [23]. Once in the *cis*-Golgi network, trimming and modifications of the glycan occurs and continues as the protein is processed towards the *trans*-Golgi network [24]. Modification of the enzymes involved in glycan processing is one possible mechanism to influence the glycan composition of therapeutic antibodies as is altering the media composition and growth conditions, as will be further discussed below. Remodeling antibody glycans after expression (termed *in vitro* glycoengineering) is also another possible approach but this requires modification to the respective enzymes located in the Golgi-membrane to efficiently recognize their substrates as soluble enzymes [25].

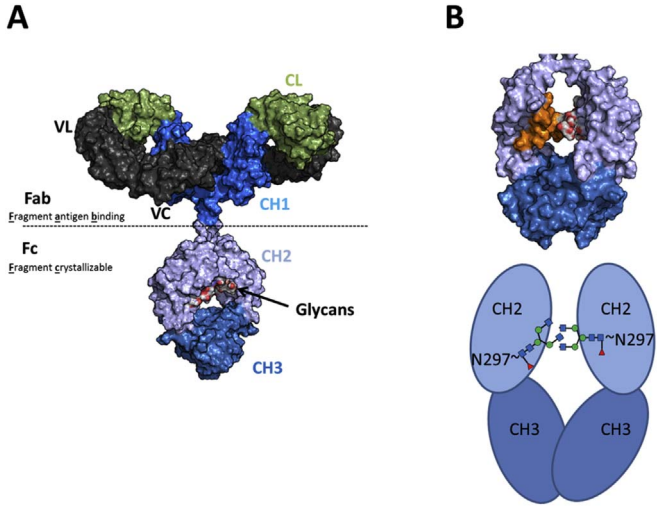

Fig. 1. IgG1 antibody structure. A) IgG-type antibodies can be divided into the Fab portion (composed of the VL and VC light chain and the CL and CH1 heavy chain domains) and the Fc portion, composed of the CH2 and CH3 heavy chain domains. Glycan residues are present in the CH2 domains of the Fc-portion. B) Enlarged view of the Fc-portion: PyMol rendered image on top shows Fc-glycans (surface-colored in orange from CH2-domain 1 and colored in grey/red from CH2-domain 2), which can interact with each other and thereby influence CH2-domain conformations. Bottom image shows a schematic illustration of the Fc-portion and indicates Fc-glycans linked to N297. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Early glycosylated forms of proteins that remain unprocessed often contain high amounts of terminal mannose residues and can contribute to the heterogeneous mixture produced in glycosylated therapeutic antibody cell culture. These forms can follow rapid clearance pathways once in the circulatory system via mannose binding receptor (belonging to the protein family of C-type lectins) on sinusoidal endothelium cells in the liver [26]. The function of this receptor is to clear pathogenic microorganisms and glycoproteins with high mannose structures. As such, mannose containing Fc-glycans need to be monitored and minimized during the production of therapeutic antibodies [27–29]. After glycan-processing (and assembly) is completed in the *trans*-Golgi network, proteins are secreted by vesicles into the extracellular space. The most prevalent glycan structures found in antibodies produced by (mammalian) cell culture are listed in Table 1. Further information on N-glycosylation has been thoroughly addressed in a recent review on the topic [30].

3. Factors that influence the glycan pattern of therapeutic antibodies

Nearly all therapeutic mAbs are produced using mammalian cells including Chinese hamster ovary (CHO) as one of the most prevalently used cellular platforms. Other mammalian cell lines are also used and the glycan pattern of the respective antibody is affected accordingly: e.g. mouse cells (SP2/0 and NSO) produce alpha 1,3-galactose and N-glycolylneuraminic acid (NGNA) on their Fc glycans [31,32]. Glycan patterns are very variable and can be influenced by many parameters of the production process, as a result production of a consistent glycan pattern continues to be a very challenging issue. Studies showing some degree of control of the glycosylation of therapeutic proteins have been recently reviewed [9,33–36]. Besides the host cell-line being the most important, other parameters in the fermentation process that may impact the final glycan patterns of therapeutic antibodies include glucose content, dissolved oxygen, bioreactor pH, sodium butyrate, ammonia content, pCO2, temperature and production scale [35].

Since so many different factors can influence the glycan pattern of a therapeutic antibody product, glycoengineering approaches have been developed to minimize the variability. Glycoengineering of mAbs is a process that is used to produce antibodies with optimized or nearly homogenous glycans by manipulating the host synthetic pathways or *in vitro* chemo-enzymatic glycan remodeling. Several other methods have been reviewed recently [37]. To date, glycoengineering has been primarily used to manufacture antibodies lacking core fucose in their glycans [37], but additional methods like inclusion of DNA encoding for glycosyltransferases to the transfection mix or co-transfection of galactosyl- and sialyltransferases to enrich certain glycans are currently emerging [38].

For increased afucosyl-content, the therapeutic antibody can be expressed in a fucosyltransferase-deficient CHO cell line. Having applied this technology, both FUT8 alleles are disrupted by homologous recombination, which results in completely afucosylated recombinant IgGs [39]. Another possibility is to use yeast as the expression system. Yeast genes that encode for enzymes producing high mannose glycans are deleted and genes that mediate the production of human-like glycostructures are inserted. Furthermore, because yeast is not able to synthesize GDP-fucose, it can be applied to produce antibodies lacking fucose in their glycans [40]. Glycosylation variants may also be engineered by a range of modifications to the oligosaccharide biosynthesis pathways. Overexpression of N-acetyl-glucosamine-transferase III (GnTIII) in the production cell line generates bisecting oligosaccharide structures and suppresses fucosylation [41]. By using in-vitro glycoengineering, after the fermentation process, nearly fully galactosylated and sialylated Fc-glycans could be produced [42]. *In vitro*