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NOTE

Faster in vivo clearance of human embryonic kidney than Chinese hamster ovary cell derived protein: Role of glycan mediated clearance

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This investigation used in vivo and in vitro tools to study pharmacokinetics and glycosylation of two monomeric antibodies produced either transiently by HEK293 cells or stably by Chinese hamster ovary cells, and demonstrated that higher in vivo clearance of human embryonic kidney antibody was due to higher glycosylation, thus higher mannose receptor mediated uptake.

[**Key words:** Antibody; Glycosylation; Pharmacokinetics; Clearance; Mannose receptor; Mannan]

Antibody therapeutics, especially immunoglobulin G (IgG) and Fc-fusion proteins have emerged as an important class of therapeutics due to their specificity, longer half-life and safety. IgG is a tetrameric glycoprotein that has two identical Fab domains connected by a dimeric Fc domain. The Fc region of IgG has been implicated in antibody's prolonged half-life through the neonatal Fc receptor (FcRn) recycling pathway (1,2). In addition to FcRn protection, there are a number of factors that can impact the pharmacokinetics (PK) of antibodies, such as the structural and physiological characteristics of the antibody, target expression and biology and proteolytic catabolism (3). Manipulating antibody charge, size, valence, FcRn binding affinity, and degree and type of glycosylation can influence antibody clearance, distribution, and lymphatic absorption (4,5).

Glycosylation are enzymatic processes where oligosaccharides (glycans) are added to specific amino acids in the polypeptide chain. Glycosylation was long considered as an unimportant protein decoration, it is now clear that it has important functions. Glycosylation affects protein folding, stability, solubility, protein–protein interactions and in vivo pharmacokinetics and immunogenicity (6–8). In general, glycans in the Fc domain are important for specific effector functions. All human IgGs contain carbohydrates at an asparagine residue in the consensus sequence of Asn-X-Ser/Thr, where X is any amino acid except proline (9). The *N*-glycans are structurally heterogenous containing a combination of terminal galactose and *N*-acetylglucosamine (GlcNAc) residues. The Fc-fusion proteins usually are engineered to join a functional polypeptide chain with Fc domain of an IgG. Depending on the sequence of the polypeptide chain, the Fc-fusion protein usually has multiple glycosylation sites. The role of glycans in protein clearance is not well understood and to some extent controversial. Glycans are usually located in the interior surface of the Fc domain, making them inaccessible to asialoglycoprotein and mannose receptors (MR) that could recognize the moieties and mediate the degradation of the antibody (5). It was observed that a wild-type and aglycosylated chimeric IgG1 showed similar PK profile in murines (5). In a recent study, high mannose (Man5) showed increased clearance in humans (4). However, Omalizumab, an IgG1 that had high mannose content, did not alter clearance in mice, yet this could be due to the fact that only a small percentage of the antibody contained high mannose (10). A reverse correlation between terminal GlcNAc (tGlcNAc) content and half-life of a protein was reported with MR viewed as the main cause of clearance of the protein (11).

Most recombinant biotherapeutics are produced in various cell culture based mammalian expression systems. It is well known that the glycosylation structures are different between mammalian and other expression systems such as yeast and insect cells (12). However, it is much less known what differences exist between mammalian cell lines. Two frequently used mammalian cell lines are HEK and CHO. HEK is a cell line originally derived from human embryonic kidney tissue. HEK cells are easy to grow and transfect, and transient transfection frequently gives good expression levels, which has made this cell line widely used in research. CHO is derived from Chinese hamster ovary. This cell line is frequently used for expression after stable transfections, it has a good long-term stable gene expression with high expression levels. For transient expression CHO is more difficult to transfect and the expression levels are frequently lower compared to transiently transfected HEK cells (13). A recent investigation has extensively studied the difference in protein glycosylation derived from HEK and CHO cells line (14). It was reported that in general, the proteins expressed in CHO cells had more acidic isoforms which is mostly sialic acid. However, no comparison of in vivo PK was reported for proteins derived from these two cell lines.

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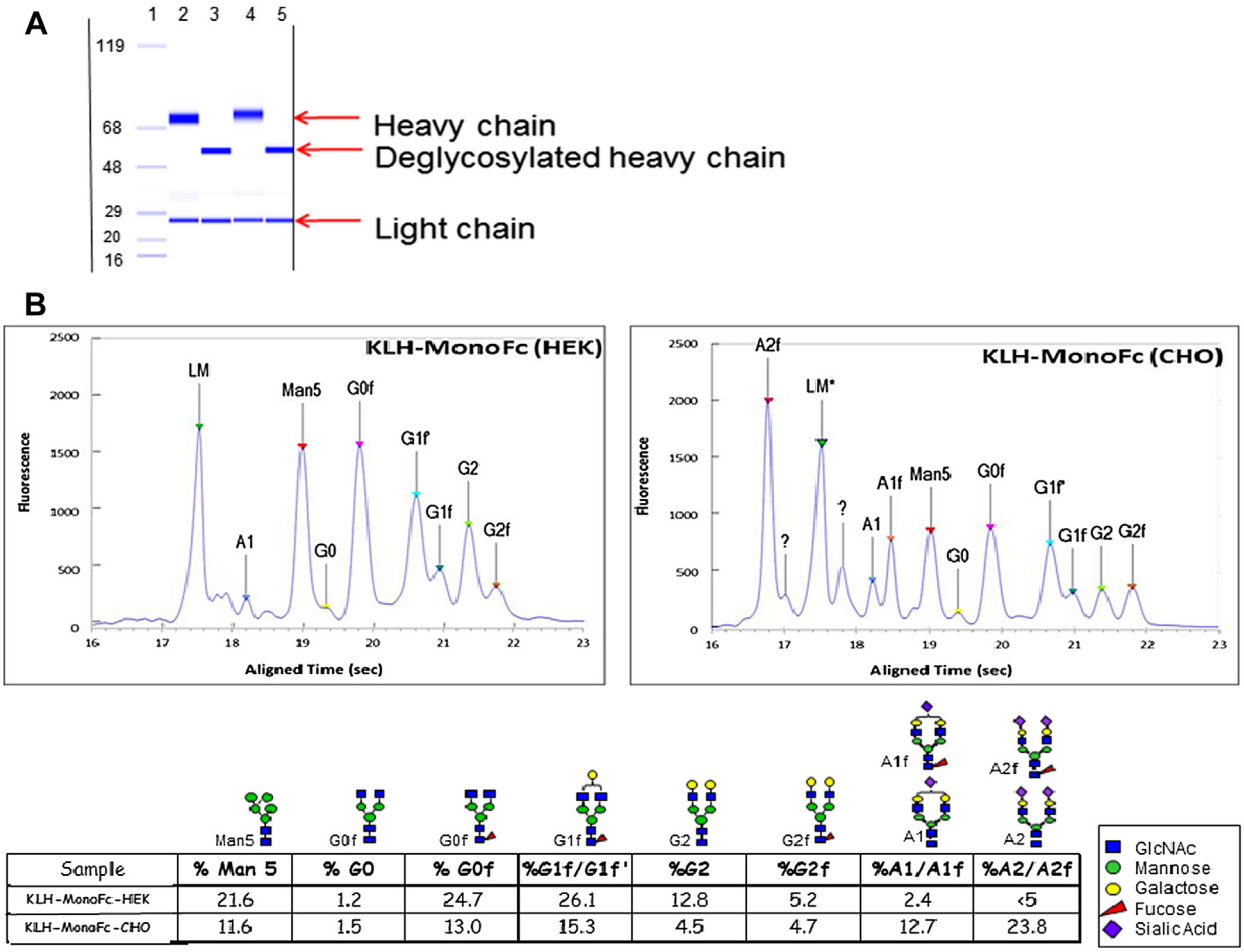
In this study, a monomeric antibody keyhole limpet hemocyanin (KLH) -monoFc was derived from both HEK and CHO cells (15). The monomeric antibody was engineered by mutational sites for *N*-glycosylation incorporated onto the C_H3—C_H3 interface of IgG that enabled conversion of dimeric Fc domain to a monomer (15). Reducing capillary gel electrophoresis (rCGE) was used to verify the KLH-monoF expressed in HEK and CHO cells are glycosylated (Fig. 1A). Briefly, proteins were analyzed under reducing condition using Perkin Elmer LabChip GXII (Hopkinton, MA). Deglycosylated KLH-monoFc samples were prepared by incubating the protein with Glycannase F (ProZyme) for 3 h at 37°C in PBS buffer. Samples for the Caliper assay were prepared according to manufacturer's instruction. Protein loading, separation, staining and destaining were performed on a quartz chip photo-etched with microchannels according to the LabChip Protein Express program. An electropherogram was generated for each sample and analyzed using LabChip GX v.3.0 software. The heavy chain from the CHO cell line was detected to have slightly higher molecular weight. After deglycosylation treatment, the heavy chain migrated at a significant lower molecular weight closer to the theoretical value of 50 kDa, and the difference between the two cell lines disappeared. There was no significant difference detected in light chain comparing CHO and HEK cell line and before and after deglycosylation. In general, IgGs are glycosylated predominantly in their heavy chain within the Fc region (16). Our results that deglycosylation treatment changed the migration of heavy chain to a much lower molecular weight has confirmed that the apparent molecular weight of the heavy chain and the observed molecular weight difference in heavy chains between CHO and HEK derived proteins are due to glycosylation.

The *N*-linked glycans profile for KLH-monoFc was conducted using the ProfilerPro Glycan Screening Assay from Perkin Elmer (Hopkinton, MA, USA) (17). This assay is analogous to the HPLC based separation of 2-aminobenzamide labeled glycans widely used in the biotechnology industry but in a high-throughput capillary microchip format. Protein samples were reduced and denatured at 70°C for 10 min prior to *N*-glycannase treatment at 37°C for 3 h. The released glycans from KLH-monoFc and a panel of commercially available glycan standards (QA-Bio) were labeled with the fluorescence dye provided in the kit at 55°C for 2 h. The labeled glycans were resuspended in deionized water, centrifuged and applied onto the HT High Resolution Chip for electrophoretic separation of the different glycan species. The resulting electropherograms were analyzed using the LabChip GX software v3.0 and the type of glycans released from KLH-monoFc was determined by comparing its electrophoretic migration time to that of the glycan standards. It has revealed that the HEK derived protein has 1.7—2.8 folder higher mannose and GlcNAc contents, but a 5 fold lower sialylated glycans than the CHO material (Fig. 1B), which is consistent with the recent report comparing protein glycans derived from HEK and CHO cells (14). In the reports, the authors argued that the difference on glycosylation patterns could be attributed to the difference on expression pattern of glycosylation enzymes between HEK and CHO, although there is no clear evidence.



Sample	% Man 5	% G0	% G0f	%G1f/G1f'	%G2	%G2f	%A1/A1f	%A2/A2f
KLH-MonoFc-HEK	21.6	1.2	24.7	26.1	12.8	5.2	2.4	<5
KLH-MonaFc-CHO	11.6	1.5	13.0	15.3	4.5	4.7	12.7	23.8

FIG. 1. (A) Capillary gel electrophoresis. From lane 1 through 5 are protein marker, KLH-monoFc-HEK, deglycosylated KLH-monoFc-HEK, KLH-monoFc-CHO and deglycoylated KLH-monoFc-CHO, respectively. (B) *N*-linked glycan analysis.