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Literature Review: CHO versus HEK Cell Glycosylation

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Abstract

Choosing a host system for the expression of recombinant proteins should be carefully evaluated prior to the initiation of any biotherapetuic development programs. Since different hosts express proteins with different efficiencies and with different post-translational modifications, changing hosts during the course of development may impact the expected activity of the protein and may require repeated studies or additional process development, delaying the time to market. Scientists must also consider the expression system's productivity, consistency, and the current regulatory environment. This paper includes a commentary on maintaining the same host system throughout the development process, lays out a brief review of key references examining the differences in glycosylation in HEK versus CHO cells, and provides a more in-depth list of related literature.

One of the most common forms of post-translational modifications is glycosylation. Half of all human proteins, and 40% of all approved therapeutic proteins, are estimated to be glycoproteins.[1,2] Glycosylation is a complex, enzyme-directed process that links oligosaccharides to specific amino acid sites. Each potential glycosylation site may be occupied or unoccupied (macroheterogeneity) and, if occupied, may contain one of a variety of glycan structures (microheterogeneity). Thus, glycosylation produces a mixed array of different protein glycoforms rather than a single homogeneously glycosylated protein.

Glycosylation impacts a large number of protein attributes ranging from folding, stability, solubility, and protein-protein interactions to more physiological properties such as protein activity, *in vivo* bioavailability, biodistribution, pharmacokinetics, and immunogenicity.[3-9] Accordingly, changes to the pattern of glycosylation can alter key therapeutic properties of a candidate molecule such as its efficacy, safety, half-life, and manufacturability.[10-14] This suggests that data generated during preclinical development may not be indicative of the final therapeutic product if glycosylation is not considered. This in turn may translate to the progression of irrelevant candidates or overlooking promising candidates, both of which negatively impact the identification of the right drug candidate.

Antibodies, the most prevalent category of biotherapeutic under development, are glycosylated on the Fc region and to a lesser degree on the Fab region.[15,16] Glycosylation is known to be a significant source of monoclonal antibody (MAb) heterogeneity. Numerous studies link this heterogeneity to changes in the biological, pharmacological, and physiochemical properties of antibodies.[17-20]

Influences on Glycosylation Patterns

A variety of factors are known to influence protein glycosylation including the protein amino acid sequence itself, the host cell, and cellular growth.[21-25] Additionally, changes to cell culture conditions ranging from culture stirring speed to nutrient availability can impact protein glycosylation.[16]

With regard to host cell-specific effects on glycosylation, it is generally accepted that bacteria, yeast, and mammalian cells differ in the level and type of post-translational modifications expressed. Unfortunately, many researchers assume all mammalian cells produce proteins with similar human-like post-translational modifications. In a key study, species-specific differences in glycosylation were observed when a MAb was expressed in cells from 13 different species, including humans.[26] All 13 species expressed the protein of interest with a heterogeneous array of biantennary complex type oligosaccharides. Variations

in core fucosylation and terminal galactosylation between species were noted in addition to significant variations in oligosaccharides sialylation.

A large number of mammalian cells derived from different species and different tissues have been successfully used to express recombinant proteins for clinical applications, including myeloma (NS0 and SP2/0) cells, Chinese hamster ovary (CHO) cells, human retina-derived cells (PerC6) cells, human embryonic kidney (HEK) cells, and baby hamster kidney (BHK) cells.[15,21] CHO cells have well established advantages for biomanufacturing including the capacity for high-level production of heterologous proteins; the ability to be cultured at high cell densities in chemically defined, protein-free media; and a strong regulatory track record. They continue to be the dominant system of choice for biomanufacturing of clinical-grade biotherapeutics. As of 2012, 70% of all recombinant therapeutics are produced using CHO cell lines.[31]

CHO versus HEK Therapeutic Protein Production

Initial efforts using CHO-based transient production of proteins were limited by poor transfection efficiencies, viabilities, and production of insufficient quantities of recombinant proteins. This has led to the use of HEK-based transient systems, which historically have higher transfection efficiencies and protein production capabilities. Multiple studies, however, have reported differences in the glycosylation patterns of proteins and antibodies when produced in CHO versus HEK cells.[32-38]

Five key studies have demonstrated that CHO cells produce proteins with higher molecular weights that can be attributed directly to differences in glycosylation (both site occupancy and associated glycostructures) and consistently contain higher sialic acid contents. This suggests that preclinical development in HEK cells increases the risk of late-stage developmental failures due to potential alterations in biophysical properties upon transition to CHO-based stable expression. Thus, if CHO cells are the intended means of biomanufacturing, the most relevant candidates will be identified using CHO cell-based protein production during early development.

Future of CHO Cell Protein Production

CHO cells lack several glycosylation enzymes present in human cells. Does this mean CHO cells produce therapeutics with suboptimal efficacy? To date, there is not a consensus on the 'ideal' pattern of glycosylation for optimal *in vivo* efficacy. Research has linked specific glycopatterns to therapeutic characteristics of antibodies including *in vivo* effector functions such as more effective antibody-dependent cell-mediated cytotoxicity (ADCC) or reduced complement-dependent cellular cytotoxicity (CDC).[39-41] While some of the identified glycopatterns may be universally beneficial, for example increased protein half-life due to increased sialylation, the optimal glycosylation state will most likely be protein-dependent, as each therapeutic protein exhibits different mechanisms of action and bioavailability. Consequently, care must be taken during candidate identification and lead optimization not only to assess protein glycosylation, but also to systematically examine the effects of specific glycosylation patterns on therapeutic efficacy.

The CHO genome project has vastly increased our understanding of CHO glycosylation pathways. Researchers have applied this knowledge to genetically engineer CHO cells to inhibit and/or express specific genes involved in glycosylation, enabling production of proteins with the desired glycopattern.[42,43] Advances in glycoengineering such as these, and the strong regulatory track record of CHO usage, support the continued use of CHO cells for the biomanufacturing of therapeutic proteins. Given the evidence that the type(s) and pattern of glycosylation are cell-type dependent and the pressures biopharmaceutical companies are under to reduce late-stage attrition rates, scientists must carefully consider the implications of switching host cell backgrounds during development.

Key Reference Review

Five recent studies examining the glycosylation of

proteins expressed side-by-side in CHO and HEK cells are summarized below. While the proteins expressed and the specific type of glycoanalysis differed in each paper, the authors of each article concluded that protein glycosylation was host cell-type dependent.

Differences in the Glycosylation of Recombinant Proteins Expressed in HEK and CHO Cells. *J. Biotech.* 2012, 161: 336-348.

The authors of this paper concluded that proteins expressed in CHO and HEK cells had significant differences in glycostructures, including a consistently increased number acidic isoforms in CHO cells due to a higher level of sialylation.

Glycoanalysis of 12 different proteins transiently expressed in CHO-S, HEK293 EBNA, and HEK 6E cell lines was conducted. The expressed proteins differed in size (9.5-52 kDa), the number of potential *N*-linked and *O*-linked glycosylation sites, and expression levels. Secreted proteins were purified and extensively characterized using SDS-PAGE, isoelectric focusing (IEF), mass spectrometry (MS), and capillary gel electrophoresis of released *N*-linked glycans.

For all proteins expressed, clear differences were detected in both the size and the number of glycans as well as the amount of sialic acid. The differences were so significant that they were detectable as changes in molecular weight observed by SDS-PAGE analysis. Changes to the molecular weight could be attributed to altered glycosylation using deglycosylation enzymes.

The most significant differences in glycosylation patterns were seen between CHO and HEK cells. Differences between two HEK cell lines (EBNA vs. 6E) as well as those due to changes in the transfection procedure or culture conditions were minor. Neither protein size nor the level of protein expression correlated with specific glycopatterns.

Characterization of Host-Cell Line Specific Glycosylation Profiles of Early Transmitted/Founder HIV-1 gp120 Envelope Proteins. *J. Proteome Res.* 2013, 12(3): 1223-1234.

This article reports the detailed glycoanalysis of HIV-1 envelope protein, gp120, transiently expressed in CHO and HEK293T cells. The authors concluded that the general degree of fucosylation and sialylation for gp120 expressed in CHO cells was higher than compared to HEK-derived gp120 and went on to detail site-specific glycan differences.

gp120 is highly glycosylated with 23 potential *N*-linked glycosylation sites. High-resolution liquid chromatography/mass spectrometry, electron transfer dissociation, and collision-induced dissociation were used for site-specific analysis of glycosylation. gp120 purified from both CHO and HEK cells consisted of a broad spectrum of glycan structures across all the potential *N*-glycosylation sites. The glycan structures included high mannose, hybrid, and complex glycans containing multi-antennary structures with varying levels of core fucosylation and sialylation.

Occupancy of individual glycosylation sites showed a high degree of similarity between CHO- and HEK-derived gp120, but distinct differences in both *O*-linked and *N*-linked glycans were identified. Specifically, two sites, N386 and N392, in the V4 region of CHO cell-derived gp120 were populated with high mannose glycans, while they were a mixture of high mannose and processed glycans in the HEK-derived gp120.

Cell Type-specific and Site Directed *N*-glycosylation Pattern of FcϒRIIIa. *J. Proteome Res.* 2011, 10(7): 3031-3039.

The dominant factor contributing to differences in FcϒRIIIa glycopatterns in these studies was the host cell used. Previous studies determined that the carbohydrate moieties at Asn-162 are important for binding of human leukocyte receptor IIIa (hFcϒRIIIa) to the Fc region of antibodies. This paper expressed hFcϒRIIIa in HEK and CHO cells and performed site-directed carbohydrate analysis via mass spectrometry

and a multienzyme protein digest. The reported studies found that the proteins had similar sites of glycosylation but the glycostructures at a number of sites were cell-type specific, including Asn-162. Furthermore, the glycosylation pattern influenced antibody binding when accessed via surface plasmon resonance.

Transient Expression of an IL-23R Extracellular Domain Fc Protein in CHO vs. HEK Cells Results in Improved Plasma Exposure. *Protein Expr. Purif.* 2010, 71: 96-102.

These studies transiently expressed the IL-23R extracellular domain Fc fusion protein (IL23R-Fc) in both CHO-S and HEK293 cells and examined the resulting glycosylation patterns. Significant host cell-specific glycosylation was reported and shown to alter *in vivo* pharmacokinetics.

IL23R-Fc contains 18 potential *N*-linked glycosylation sites within a single dimer. Following expression, IL-23R glycopatterns were examined via SDS-PAGE, IEF, and lectin microarray binding. Additionally, the purified IL23R-Fc proteins were administered to mice and the *in vivo* pharmacokinetics analyzed. Differences in the molecular weights seen by SDS-PAGE were confirmed to be due to differences in *N*-linked glycosylation. Additionally, the IL23R-Fc produced by CHO cells exhibited a lower pI, indicating an increased number of acidic groups. Sialidase treatment and IEF analysis confirmed the decrease in pI was due to differences in sialic acid content. Compared with CHO-derived protein, that from HEK cells had increased binding to lectins specific for terminal galactose (Gal) or *N*-acetyl-glactosamine (GalNAc) residues. Overall, the authors concluded that CHO-derived protein had a higher total sialic acid content while HEK-derived protein had a higher terminal Gal content.

Despite clear differences in glycosylation, CHO- and HEK-derived proteins had comparable *in vitro* activity as measured by binding to IL-23 and stimulation of IL-18 production upon exposure to human peripheral blood mononuclear cells (PBMCs). *In vivo*, however, CHO-derived IL23R-Fc had approximately 30-fold higher plasma exposure and a 2-fold longer plasma half-life. The major difference was the very high clearance of HEK-derived IL23R-Fc from plasma within the first hour. This finding is consistent with glycoprotein receptor-mediated clearance, as carbohydrates lacking terminal sialic acid are potential substrates for glycoprotein receptor binding.

High-level Protein Expression in Scalable CHO Transient Transfection. *Biotechnol. Bioeng.* 2009, 103(3): 542-551.

This article reports the glycoanalysis of a monoclonal antibody purified from three sources: 1. transiently expressed in HEK293 EBNA cells, 2. transiently expressed in CHO-K1SV cells, and 3. purified from a CHO-derived stable cell line. The authors concluded that the glycan distribution of HEK-derived antibody was dramatically different than CHO-derived antibodies when examined via reverse phase liquid coupled with electrospray time-of-flight chromatography.

The distribution of major CHO glycoforms was comparable between transiently and stably expressed antibodies. These data support the use of transient expression as a means of protein production during therapeutic development, even for proteins that will ultimately be manufactured using stable cell lines, if the cell background is maintained.

Further Reading

Relevant Review Articles

The Choice of Mammalian Cell Host and Possibilities for Glycosylation Engineering. *Current Opin. Biotechnol.* 2014, 30: 107-112.

Application of Quality by Design Paradigm to the Manufacture of Protein Therapeutics. *Glycosylation.* Dr. Stefana (Ed.). 2012, DOI: 10.5772/50261. Available from: http://www.intechopen.com/books/glycosylation/application-of-quality-by-design-paradigm-to-the-manufacture-of-protein-therapeutics

Towards the Implementation of Quality by Design to

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Glycosylation of Therapeutic Proteins in Different Production Systems. *Acta Paediatr. Suppl.* 2007, 96(455): 17-22.

Additional Comparison of CHO versus Human-Cell Line Glycosylation

Erythropoietin Produced in a Human Cell Line (Dynepo) Has Significant Differences in Glycosylation Compared with Erythropoietins Produced in CHO Cells. *Mol. Pharm.* 2011, 8: 286-296.

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Protein Glycosylation in CHO Stable versus Transient Expression

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