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Native Glycosylation Is Important For Biological Function and Activity of Recombinant Human Proteins

INTRODUCTION

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SUMMARY

Although recombinant human cytokines are commercially available from bacterial, eukaryotic, and mammalian cell expression systems (*E. coli*, SF9, CHO), human cell-expressed (HEK293) glycoproteins offer the distinct advantage of native human glycosylation. In this Whitepaper, several examples illustrating the importance of correct glycosylation in cytokine activity, stability and half-life, are provided for cytokines widely used in stem cell research.

INTRODUCTION

Cytokines are glycoproteins of the immune system that act as cellular signaling molecules. Most cytokines are less than 30 kDa in size and bind to specific, high-affinity cell surface receptors, leading to changes in the rate of cell proliferation and/or in the state of cell differentiation. Cytokines are widely used in research, diagnostics and therapeutics, and there exists an increasing demand for high quality recombinant human cytokines for stem cell and clinical research.

Glycosylation describes the addition of a carbohydrate moiety (glycan) to proteins after translation, and can be *N*-linked with glycans added to the nitrogen of asparagine (Asn) or arginine (Arg) side chains, whereas O-linked glycans attach to the hydroxyl oxygen of serine (Ser), threonine (Thr), or tyrosine (Tyr) side chains. It is well established that addition of the correct carbohydrate chain(s) to a complex glycoprotein affects its structural and functional properties including conformation, receptor binding, intracellular transport and secretion, solubility, susceptibility to proteases and *in vivo* half-life.

However, recombinant human cytokines, many of which are glycosylated, are predominantly commercially produced in non-human cells (e.g. *E. coli*, SF9, CHO). While these expression systems are favored for their ability to produce large quantities of recombinant protein, there are key differences in the glycosylation machinery of lower organisms (that have essentially no glycosylation machinery) non-human eukaryotic or mammalian cells, and human cells. Proteins from non-human cell expression systems, whose glycosylation patterns differ from the native human proteins, lack authenticity and physiologically relevant glycosylation. It is clear that human recombinant cytokines expressed in a human cell line (HEK293; human embryonic kidney) with native glycosylation offer unique and significant advantages over non-human expression systems.

Erythropoietin

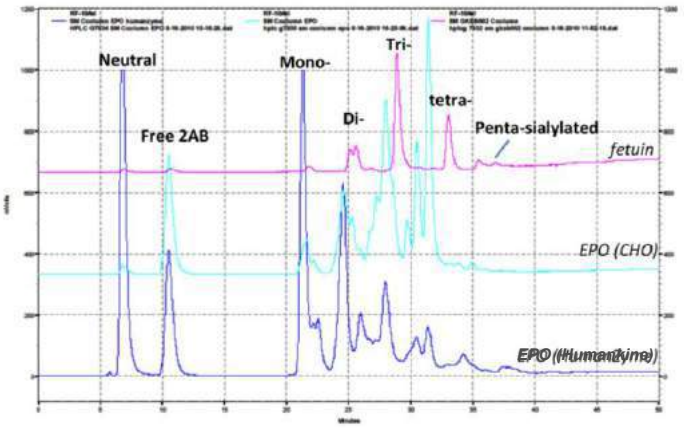
Erythropoietin (EPO) is a 166-amino acid 34 kDa cytokine hormone that stimulates the production of red blood cells in bone marrow, and is used in stem cell research for the proliferation and differentiation of endothelial progenitor precursor cells and other cell types.

EPO is a glycosylated protein whose oligosaccharides are added to the nascent peptide backbone during translation at 4 glycosylation sites (Asn24, Asn38, Asn83, Ser126). These glycan chains are then further processed and edited in the Golgi apparatus, producing a complex glycan structure comprising 40% of the secreted protein's weight, where each N-linked glycan tree consists of 2, 3, or 4 branches with a terminal sialic acid.

This complex glycosylation pattern is essential to the activity of EPO; the composition and degree of branching of these oligosaccharides have been shown to modulate the pharmacodynamics, degradation, stability, solubility and biological activity (Skibelli, 2001 and Egrie/Brown, 2001), and EPO with no glycosylation is completely inactive.

Commercially available EPO is expressed in mammalian cell lines, primarily Chinese hamster ovary (CHO). However, the glycosylation patterns of proteins expressed in CHO cell lines are known to have significant differences in the pattern and composition of glycan structures compared to human cell

Figure 1. HPLC Analysis of Human vs CHO EPO Glycans



lines (HEK293), including the size and number of the glycostructures and the amount of sialic acid in the glycan trees (Croset, 2012).

Figure 1 shows a comparison of HEK293- and CHO-expressed EPO prepared by fluorescent labeling and release of the glycan structures, followed by HPLC separation. Calf serum fetuin (a glycoprotein containing sialylated *N*-linked and *O*-linked glycans) was used as a positive control for endoglycosidase enzymes. The results show that Human EPO contains a large number of neutral glycans, with mostly mono-sialylated and smaller amounts of di-, tri-, tetra-, and penta-sialylated glycans. The CHO-expressed EPO contained more highly sialylated glycans, with smaller amounts of neutral glycans. A study by Skibeli et al (2001) also supported the predominance of neutral, mono- and di-silated glycans in human EPO isolated from human urine, and more highly silated EPO in CHO-produced recombinant protein.

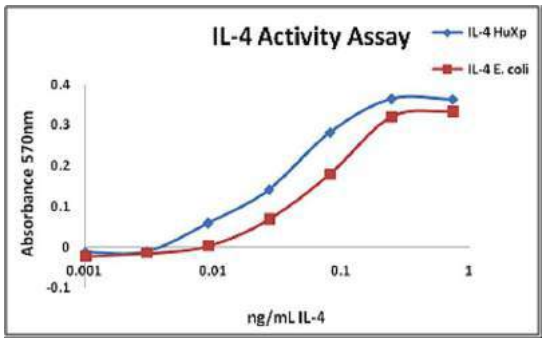
Given the heterogeneity of the type of glycans found in the human and CHO EPO, and the evidence that the Proteintech Humankine EPO has native human glycosylation patterns, EPO expressed in a human cell line with the authentic human glycan structure intact should be used for in vitro research applications wherever possible.

IL-4

Interleukin 4 (IL-4) is produced by activated T cells, mast cells, basophils and eosinophils. This cytokine has numerous B cells, T lymphocytes, monocytes, macrophages, mast cells, fibroblasts, functions such as differentiation of endothelial cells, and other cell types. IL-4 plays a critical role in the development of allergic inflammation and asthma.

IL-4 has 129 amino acids, with two possible sites for N-linked glycosylation and 6 cysteine residues forming three disulfide bonds. Its molecular weight is between 12 and 20 kDa depending on natural glycosylation. *E. coli* expressed IL-4 has an apparent molecular mass of 14 kDa, compared with IL-4 produced from a man cell line, which migrates as a major band of 19 kDa due to native human glycosylation.

Figure 2. IL-4 Activity Assay for IL-4 From HEK293 and *E. Coli*



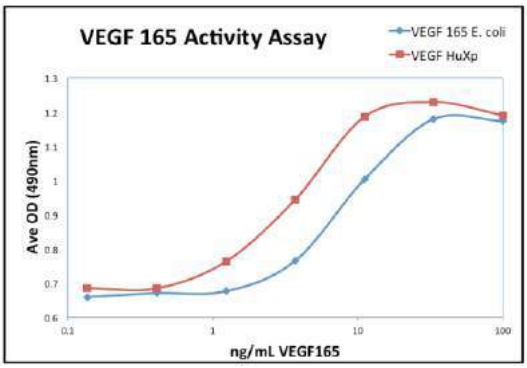
To investigate whether the native glycosylation of IL-4 expressed in human cells shows an advantage over E. coli produced, the dose-dependent stimulation of the proliferation of human TF-1 cells was determined at 37ºC. As shown in Figure 2, IL-4 from HEK293 has 5-fold higher potency, demonstrating that the authentic human glycosylation offers superior activity over the E. coli expressed cytokine.

VEGF 165

Vascular endothelial growth factor (VEGF165) is a member of the cysteine-knot growth factor superfamily. This cytokine stimulates proliferation and survival of endothelial cells, and promotes angiogenesis and vascular permeability. Expressed in vascularized tissues, VEGF plays a prominent role in normal and pathological angiogenesis.

VEGF is a homodimeric glycoprotein with an apparent molecular mass of 45 kDa due to glycosylation at one glycosylation site and its dimerization. Glycosylation of VEGF is important for efficient secretion, however the role of glycosylation in VEGF165 biological activity is unclear. Figure 3 indicates VEGF165 from HEK293 is 3-fold more active than the *E. coli* expressed protein.

Figure 3. Activity Assay for HEK293 and *E. coli* VEGF165



The bioactivity of VEGF165 was determined by its ability to induce proliferation of human umbilical vein endothelial cells. This supports the importance of correct glycosylation in the biological properties of VEGF165 in cell culture.

GM-CSF

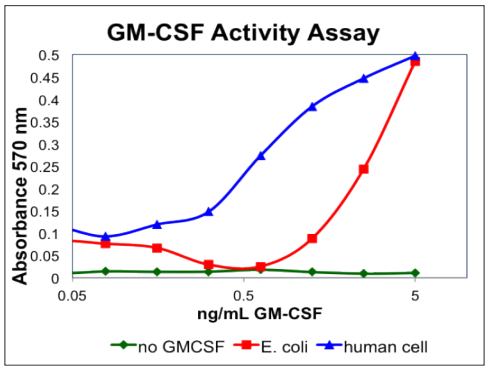
Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor that stimulates the development of neutrophils and macrophages, and promotes the proliferation and development of early erythroid megakaryocytic eosinophilic progenitor cells. GM-CSF is produced by several different cell types including activated T cells, B cells, macrophages, mast cells, endothelial cells and fibroblasts in response to cytokine, immune and inflammatory stimuli.

GM-CSF is a monomeric 127 amino acid glycoprotein with two disulfide linkages that migrates as a broad band of 15-30 kDa due to glycosylation and sialylation.

N-linked glycans are located at Asn27 and Asn37, and multiple potential sites for 0-linked glycosylation exist at at Ser5, Ser7, Ser9 and Ser10 but the extent of glycan structures at these sites have not been unambiguously determined (Zhang, 2014). The glycosylation patterns of GM-CSF have however been observed to influence its activity, receptor binding, immunogenicity, and half-life (Cebon, 1991).

To evaluate the biological activity of GM-CSF produced from HEK293 cells compared to the unglycosylated form produced from *E. coli*, the dose-dependent stimulation of the proliferation of human TF-1 cells was determined for both sources. Both versions of GM-CSF were assayed for cell proliferation (MTT assay) at 37°C for seven days. As shown in Figure 4, GM-CSF from HEK293 has 10-fold higher potency than *E. coli* expressed cytokine, demonstrating that native human glycosylation of GM-CSF has superior activity and greater stability in cell culture conditions.

Figure 4. Activity Assay for GM-CSF From HEK293 and *E. coli*



SUMMARY

Native human glycosylation was shown to play a key role in the activity and stability of EPO, IL-4, VEGF165, and GM-CSF. Correctly glcosylated recombinant human cytokines from a human cell line (HEK293) offer unique advantages over proteins produced in *E. coli* or CHO cells, including superior stability and activity in cell culture. These advantages are important to consider when choosing recombinant human cytokines for research applications.

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