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Research Paper

A low redox potential affects monoclonal antibody assembly and glycosylation in cell culture

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ABSTRACT

Glycosylation and intracellular assembly of monoclonal antibodies (MAbs) is important for glycan profile consistency. To better understand how these factors may be influenced by a lower redox potential, an IgG1-producing NS0 cell line was grown in the presence of varying concentrations of dithiothreitol (DTT). Cultures were monitored for growth and culture redox potential (CRP) with glycan heterogeneity determined using a HILIC-HPLC method. Macroheterogeneity was unchanged in all conditions whereas the Galactosylation Index (GI) decreased by as much as 50% in cultures with lower CRP or higher dithiothreitol levels. This shift in GI is reflected in more agalactosylated and asialylated species being produced. The MAb assembly pathway was determined using radioactive isotope ^{35}S incorporated into nascent IgG1 molecules. The assembly pathway for this IgG1 was shown to progress via HC → HC_2 → HC_2LC → HC_2LC_2 in all conditions tested and autoradiographs highlighted that the ratio of heavy chain dimer to heavy chain monomer increased over time with increasing DTT concentrations. This increase and correspondingly lower GI values may be due to disruption of the disulfide bonds at higher levels of assembly. A change in the assembly pathway may alter the final IgG glycan pattern and lead to control mechanisms that influence glycan profiles of MAbs.

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1. Introduction

The impact of variation in *N*-glycosylation on therapeutic glycoproteins is significant, with changes that can affect protein folding, stability, pharmacokinetics and immunogenicity (Jefferis, 2009). This ultimately determines their efficacy and ability to induce cell-mediated responses such as Antibody Dependent Cell Cytotoxicity (ADCC) and Complement Dependent Cytotoxicity (CDC), thus making it essential for consistent *N*-glycan profiles. The control of parameters that affect glycosylation during the cell culture process is important to reduce batch-to-batch variability as well as to enhance the production of glycoforms identified with desirable activities. As a result, many studies have been performed to identify and manipulate the culture parameters that affect glycosylation patterns of recombinant glycoprotein (Hossler et al., 2009) and to derive the desired glycan structures. A number of bioprocess parameters contribute to variation in glycosylation profiles: expression/production systems, Dissolved Oxygen (DO) concentration, pH, Culture Reduction Potential (CRP), ammonia levels and nutrient depletion (Gawlitzek et al., 2000; Kunkel et al., 1998; Muthing et al., 2003; Yang and Butler, 2000). The effect of variable concentration of DO has been studied extensively. Lowering the DO concentration has been shown to decrease the extent of galactosylation in MAbs with little to no effect on MAb productivity. It has been shown that when DO was decreased to 10% in a chemostat culture of hybridoma cells there was an increase in the production of agalactosylated MAbs compared to cultures maintained at 50% or 100% DO (Kunkel et al., 1998). One explanation for this effect is that the low DO caused a shift in the intracellular redox balance with a consequent effect on cellular metabolism. When DO is lowered in cell culture, a shift in the culture redox potential (CRP) may disrupt disulfide bonds, which may have a profound effect on the synthesis of immunoglobulins, and their carbohydrates, because of the structural proximity of the consensus glycosylation site to an interchain disulfide bond. A disturbance in disulfide bond formation may

Abbreviations: MAb, monoclonal antibody; ADCC, antibody dependent cell cytotoxicity; CDC, complement dependent cytotoxicity; DTT, dithiothreitol; DO, dissolved oxygen; CRP, culture redox potential; tPA, tissue plasminogen activator; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HILIC-HPLC, hydrophilic interaction liquid chromatography – high pressure liquid chromatography; RIPA, radio-immunoprecipitation assay; β-ME, β-mercaptoethanol; GalT, β-1,4 galactosyltransferase.

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cause a change in the accessibility of galactosyltransferases and subsequent effect on galactosylation.

In another study it was shown that an oscillating DO, that mimics what may occur in heterogeneous large scale manufacturing runs, does indeed influence the overall glycan microheterogeneity (Serrato et al., 2004). As cultures were exposed to an oscillating DO, the transitory periods between low and high oxygen availability enhanced sialylation and tri-antennary structures. A proposed explanation for these results is that the oscillations caused a shift in the internal redox balance of the cells thereby altering the glycan pathway. Although CRP was not measured directly, the effects of a shifting DO environment could be related to metabolic changes under reducing conditions. This reducing environment may be responsible for altering both the cell's protein folding mechanisms and glycan processing.

Unlike pH or DO, CRP is not a parameter that is measured routinely and is thus not very well understood, although attempts have been made. It has been seen that when the CRP was controlled at specific values and compared to cultures maintained at constant DO (Meneses-Acosta et al., 2012). The findings indicated that reducing conditions (redox <0.0 mV) enhanced cell culture performance with respect to specific growth rate and overall MAb concentration although no glycan analysis was performed to determine product quality. It was determined that control strategies involving CRP could be employed to maximize cell growth and productivity by shifting CRP during cell culture operations.

It has been reported that the intracellular redox state of mammalian cells has an impact on protein folding and disulfide bond formation during monoclonal antibody production (Guo et al., 2008; Liu et al., 2010). The disruption of these bonds may impact glycosylation by allowing or eliminating any steric hindrances affecting access of glycosyltransferases. Although protein structures are synthesized in the endoplasmic reticulum they may be subsequently modified in the Golgi. MAbs enter the Golgi fully folded, but the possibility remains that a reducing environment may allow disulfide bond disruption and therefore alter structural access to the glycosyltransferases.

In order to better study the effect of altered redox potentials on protein synthesis in a cell culture system, a reducing agent such as DTT may be introduced into the medium. DTT has previously been shown to alter the glycan macroheterogeneity of recombinant tissue plasminogen activator (tPA) resulting in the production of primarily fully glycosylated tPA (type I) (Allen et al., 1995). The mechanism for this is proposed to be through the delayed formation of a disulfide bond that would extend the exposure of a specific glycan site. Without the disulfide bond, the oligosaccharyltransferase adds the consensus primary glycans to the nascent polypeptide chain in the endoplasmic reticulum (ER). The oxidizing environment of the ER can be altered with DTT in the culture medium resulting in misfolded proteins and therefore lower production rates but the effect can be reversed when the reducing agent is removed (Lodish and Kong, 1993). The redox environment of the Golgi is not as well understood but it is likely that some change in the redox balance would affect the post-translational modifications occurring therein. A shift to a more reducing environment within the Golgi could cause changes in structure to increase or decrease the access of glycosyltransferases to any protein.

The objectives of the work presented here were to determine the effects of a reducing agent on cell culture performance, glycosylation profiles and assembly of a humanized immunoglobulin (IgG1) produced from a transfected mouse cell line (NS0). In addition, the levels of IgG1 intermediates were monitored under various reducing conditions to determine any relationship between disulfide bonding and changes in glycan microheterogeneity. The activity and effect of β-1,4 Galactosyltransferase (GalT) on GI shift under low CRP was also investigated to 1) identify if GalT was being affected by addition of DTT 2) determine if GalT had differential effect on fragments of IgG1. These results are important for understanding the effects of redox potential on the control of antibody assembly and glycosylation.

2. Materials and methods

2.1. Cell culture

A proprietary NS0 cell line (Hu1D10) provided by Abbvie (Redwood City, CA, USA), cultured in PFBM-1 (protein free basal media, Abbvie; 15 g/L glucose, starting pH = 7.2 ± 0.1, osmolality = 300 mOsm/kg H_2O) was used for this study. This cell line has been transfected with a gene for recombinant humanized IgG1 and is considered to be a high producer of IgG1 (titres >500 μg/ml in batch culture over 7 days). Cell passaging was performed every 3 days in T75 flasks incubated at 37 °C, 10% CO_2. Cell counts were performed using the Cedex XS cell counter (Roche, Indianapolis, IN, USA) that utilizes the trypan blue exclusion method. Cultures were seeded at 2.0×10^5 cells/ml with viabilities above 95%. DTT was added to experimental cultures prior to inoculation at 0.25, 0.50, and 0.75 mM. DTT was not added to the control cultures. All cultures were established in duplicate over multiple trials with samples removed daily for analysis. Cultures were harvested on day 4 when viabilities were >90%. The high viability at harvest minimized any protein degradation in the supernatant.

2.2. Redox potential and pH measurements

Redox potential was measured using a redox probe (9179BNMD Thermo Fisher, Waltham, MA, USA) and pH measurements with a low maintenance gel-filled pH probe (Thermo Fisher, Waltham, MA, USA). Both probes were plugged into an Orion 420A meter (Thermo Fisher, Waltham, MA, USA). The pH probe was calibrated with 4,7 and 10 pH buffers. Redox measurements were performed based on a protocol previously established (Pluschkell and Flickinger, 1995). The redox probe was set up daily prior to sampling by immersing the probe for a period of 10–15 min in preconditioned room temperature PFBM-1. This allowed for more accurate and stable redox values of samples. A 3 ml sample was removed every 24 h from each sample flask for sequential redox potential and pH measurements with as little agitation as possible while taking readings to minimize gas exchange. For best results the meter readings were allowed to stabilize over a period of 1–3 min (Meneses-Acosta et al., 2012). Samples were retained for titre determination.

2.3. MAb purification

Protein A columns or spin filters were used for Mab purification. (a) 1 ml HiTrap Protein A HP columns (GE Healthcare, Pittsburgh, PA, USA) were prepared prior to sample loading by flushing with 3 column volumes (CV) of elution buffer (0.1 M glycine pH 3.0) and equilibrated with 5 CV of washing buffer (PBS pH 7.0). The sample was loaded by syringe and washed with 5 CV of PBS. The bound antibody was stripped with 3 CV of elution buffer and the eluant collected. All flow rates varied between 1.0 and 1.5 ml/minute. The eluant was neutralized to pH 6.5–7.5 with Tris buffer pH 9.0 so as to minimize sialic acid loss. The maximum binding capacity of the column (20 mg IgG/ml) was never reached. (b) Protein A HP SpinTrap filters (GE Healthcare, Pittsburgh, PA, USA) were prepared for use by placing a filter in a 1.5 ml microfuge tube, spinning out the storage solution and resuspending the protein A media in 600 μl of binding buffer (PBS pH 7.0). Binding buffer was spun out and up to 600 μl of sample were loaded. After gently mixing for 4 min to allow antibody binding, the column was spun down and twice resuspended in 600 μl of binding buffer, spinning down each time. All filtrate was

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