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Mammalian cell-produced therapeutic proteins: heterogeneity derived from protein degradation

Haimanti Dorai and Subinay Ganguly

Therapeutic glycoproteins, for example, antibodies (Abs) and Fc fusion proteins when produced in mammalian cells, such as Chinese hamster ovary (CHO) cells generally exhibit heterogeneity. Both the oligosaccharide moiety and the protein moiety contribute to this phenomenon. Non-enzymatic and enzymatic pathways of protein fragmentation generate heterogeneity in the polypeptide backbone. In the non-enzymatic pathway, physical and chemical events such as light, oxidation, and others can cause the protein moiety to become unstable leading to its fragmentation. Intracellular and secreted proteases are involved in the enzymatic degradation of proteins. This degradative process is modulated by the oligosaccharide moiety of the glycoprotein as well as glycosidases, including sialidases that are secreted in the culture medium. This review focuses on the factors that modulate heterogeneity of the protein moiety especially by the enzymatic methods. Availability of the CHO genome database will facilitate the development of host cell lines with minimal degradative properties.

Addresses
Janssen Research and Development, LLC, Spring House, PA, United States

Corresponding author: Ganguly, Subinay (sgangul2@its.jnj.com)

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Introduction

The total annual revenue of therapeutic proteins including that of monoclonal Abs and fusion proteins is currently reaching ~$64 billion in the USA [1•]. This figure is projected to increase in the future since the number of new Abs, antibody (Ab) fragments, and protein/peptide-antibody or protein/peptide-Fc fusion proteins (referred to as Fc fusion proteins) targeted to treat diseases in oncology, immunology, and neurology [2•] that are being discovered has exploded in number. To feed these advancements, identification and development of production methods that are rapid, robust, and cost-effective is critical. Since a majority of the biologic therapeutics, including Abs and Fc fusion proteins are glycoproteins that undergo posttranslational modifications, mammalian cells are favored as the production platform. Only a small subset of biologics is expressed in other systems [see Ref. [3] for a review]. Cell lines such as Chinese hamster ovary (CHO) and mouse myeloma (Sp2/0 and NS0) are commonly employed for high-level expression of recombinant therapeutic proteins in mammalian cells [see Ref. [4••] for a review]. With the combination of high producing cell lines and optimized fed-batch processes, cell culture productivity has increased from tens of milligrams per liter in the 1990s to the multi-grams per liter level in recent years [5].

There is one negative consequence of using mammalian cell lines as the production platforms, however, and that is the products generated in mammalian cell-based production systems are of a heterogeneous nature. As the analytical tools have become increasingly sophisticated [6•], products that were previously considered homogeneous are now deemed to be heterogeneous. This heterogeneity has the potential to impact product quality and thus is a real concern from a pharmacological standpoint [7,8••]. Therefore, understanding the underlying mechanisms that cause product heterogeneity is important as this may lead to the development of methods for controlling such heterogeneity.

Factors that cause heterogeneity

Heterogeneity of biotherapeutics expressed in mammalian cells can result from a number of sources. The transfected gene [9], the primary sequence of the protein that potentiates amino acid mis-incorporation [10], and the host cell line used [11,12•,13••,14•], all has been shown to contribute towards product heterogeneity. As mentioned above, a majority of biologic therapeutics, including Abs and Fc fusion proteins are glycoproteins. Glycoproteins are comprised of two components, namely, the glycan moiety and the protein/peptide moiety. The microheterogeneity of the glycan moiety can contribute significantly towards the heterogeneity of the glycoprotein, a subject that has been discussed extensively [6•,15••,16]. This review will focus on the heterogeneity of glycoproteins generated from clipping or fragmentation of the protein/peptide moiety, especially by enzymatic methods.

Protein degradation by non-enzymatic factors

When a protein becomes unstable, degradation or fragmentation ensues. Factors that render a protein unstable

can be enzymatic and/or non-enzymatic in nature. The non-enzymatic factors that cause proteins to become unstable are either physical or chemical events. Often, these two types of events work together, for example, oxidation of a protein (a chemical process) can result in its aggregation (a physical process). For clinical applications, biotherapeutics are formulated with excipients at high concentrations at or near physiological pH. Since proteins are compositionally and conformationally stable within a narrow range of pH and osmolarity, the stability of biopharmaceutical products can be compromised by factors such as long-term storage in a container-closure system that is subjected to variations in storage temperature, exposure to light, agitation, and others leading to its aggregation and degradation. Aggregation as well as precipitation of proteins can also occur because of denaturation, dissociation, and protein unfolding/misfolding. Aggregated proteins can potentially be immunogenic in addition to being a target for degradation/fragmentation, which leads to heterogeneity [17,18]. Chemical instability results when covalent bonds are formed or destroyed. These include hydrolysis, oxidation, isomerization, reduction, deamidation, photodegradation, and disulfide scrambling. Additionally, posttranslational modification like phosphorylation, N-terminal pyroglutamine cyclization, and less common modification of the N-terminal and C-terminal amino acids [19] alters the degradative pathway and the kinetics of degradation [for review see Refs. [20,21•]]. All of these events ultimately induce the protein towards one or more non-enzymatic methods of fragmentation.

Unlike other glycoproteins, Abs are remarkably stable proteins and are resistant to many of the above-mentioned degradative pathways, with the only exception being some instability in the relatively exposed and flexible hinge region. A key set of cleavage sites in the upper hinge of human IgG1 heavy-chain is in the sequence Ser_{219}-Cys_{220}-$Asp_{2\backslash 21}$-Lys_{222}-Thr_{223}-His_{224}-Thr_{225}-Cys_{226}, (EU numbering), with the predominant site being between Asp_{221} and Lys_{222}. These sites were determined to be subjected to non-enzymatic cleavage [22]. Cleavage was found to be peptide sequence-dependent and likely to result from β-elimination and peptide bond hydrolysis. Additional mechanisms include acidic pH-induced Asp_{221} isomerization [23•,24•] and/or the involvement of hydroxyl-radical formation mediated by His_{224} [25•,26•], both of which induce cleavage of the contiguous peptide bond.

Protein degradation by enzymatic methods

Another route by which heterogeneity is generated in therapeutic glycoproteins is by proteolytic degradation. Immunoglobulins, particularly IgGs are soluble glycoproteins and are N-glycosylated at a single conserved site (Asn_{297}) in the CH2 domain of the Fc [27]. IgGs are rather unique among glycoproteins in that the glycans are not on the surface of the molecule but instead are somewhat sequestered in a cavity between the two heavy chain polypeptide backbones of the Fc [for a cartoon see Ref. [28]]. This sequestration not only helps the protein backbone to establish a defined structure for the glycans, but for the glycans to help establish a defined structure for the protein backbone. The conserved lower hinge/CH2 junction region of an Ab is comparatively more flexible and has several sites that are sensitive to enzymatic and non-enzymatic fragmentation. Papain, a non-specific sulfhydryl protease from the papaya plant, cleaves IgGs primarily in the upper hinge region to yield Fc and Fab fragments. Interestingly, aglycosylated or enzymatically deglycosylated IgGs or Fc fragments derived from such Abs could be digested into smaller fragments much faster and more completely compared to normally glycosylated IgGs or Fc, suggesting that the three-dimensional (3D) structure of Fc imparted by the Fc glycan is important for increased Ab resistance to proteases [29]. In humans, the Fc glycan is of the complex bi-antennary type with a high degree of heterogeneity due to incomplete glycosylation processing. The glycan heterogeneity impacts the proteolytic cleavage of Ab resulting in a mixture of intact Ab, Fc, and Fab fragments affecting its pharmacokinetic and pharmacodynamics properties. Since cell culture conditions are known to modulate the activities of the glycosylating enzymes [see below and for review, Ref. [8••,30]], it is imperative that the cell culture parameters are established early on during process development that will generate the desired product in a consistent manner.

In clinical setting, several proteases associated with inflammation, tumor invasion, metastasis, and bacterial infections have the ability to fragment IgGs. These proteases preferentially cleave IgGs in the lower hinge and include the matrix metalloproteinases (MMPs) such as stromelysin-1 (MMP-3) and metalloelastase (MMP-12) (both cleave between Pro_{232} and Glu_{233}), matrilysin (MMP-7) (cleaves between Lys_{234} and Lys_{235}), cathepsin G, *Staphylococcus aureus* glutamyl endopeptidase I (GluV8) (both cleave between Glu_{233} and Lys_{234}) and the IgG-degrading enzyme of *Streptococcus pyogenes* (IdeS) (that cleaves between Gly_{236} and Gly_{237}) [31••]. Proteolytic cleavage of IgGs by these proteases occurs in a step-wise process, whereby first one heavy chain is cleaved, generating an intermediate product that has a single cleavage in the lower hinge. Breszki *et al.* [31••] showed that these intermediate species do not detectably bind to Fcγ receptors nor to the complement protein C1q, and therefore are devoid of immune effector functions like antibody dependent cell mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Importantly, these single-clipped Ab species remain intact and have comparable half-life to that of intact Abs. Tumor-associated proteases whose affinity for intact Abs is significantly higher than that of single-clipped species thus

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