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GLYCOPINION

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The field of Glycobiology addresses the role of oligosaccharides in biological events. It is an interdisciplinary science which is making a vital contribution to our understanding of molecules which are of biochemical, pharmaceutical and medical interest. The rapid explosion has arisen from advances in technology which allow the release, isolation, characterization and structure determination of oligosaccharides. There is an increasing awareness that oligosaccharides modify protein functions as well as being involved in biomolecular recognition.

In this section international contributors from industry, academia and government research institutions are invited to explore the scientific and ethical issues emerging from this developing field.

The major issues raised in this first article by Dr Kenneth Seamon of the FDA, provide an opportunity for glycobiologists to address some fundamental questions including:

- In this day and age are we content to manufacture poorly characterized products?
- If products are not well defined how can we achieve high standards of quality control?
- Given that complete characterization is not a realistic goal what parameters should be used to assess biological products?
- Is heterogeneity an advantage in a drug, or are some glycoforms more efficacious than others?
- Which, if any, *in vitro* systems are suitable for testing biological products?
- Should standards of purity be defined in relation to efficacy in treating the disease, or to the biological function of the naturally occurring compound, to the *in vitro* activity or to the structure analysis of the purified glycoconjugate?
- Do we really need to understand the relationship between structure and function, or is it so complicated by heterogeneity that we are incapable of rationalizing it?

Evaluation of recombinant glycoproteins

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Recombinant technology has provided a means of producing therapeutic drugs and biologicals that had previously been obtainable only in small quantities. In contrast to conventional drugs which are primarily small molecules produced by organic synthesis, these therapeutic products are produced in living organisms. Biological synthesis necessarily invokes diversity and, therefore, biological products are composed of heterogeneous mixtures of active species that have been historically difficult to characterize. Although recombinant DNA technology has allowed the production of large quantities of therapeutic proteins in cell culture, it has not eliminated the heterogeneity and diversity that is associated with biological products.

The heterogeneity and diversity which define a biological product are determined by the process of manufacture. This process includes the nature of the host cell, the conditions of host-cell propagation, the purification strategy, and the formulation steps. All of these steps contribute to a biological diversity. Therefore the careful evaluation of the manufacturing process is essential to the review of a biological for use as a therapeutic or a prophylactic product such as a vaccine.

The primary goals in reviewing a biological product are to determine if the product is safe and efficacious and whether or not it can be produced by a well-controlled process to ensure its consistency. A framework has been developed for evaluating questions regarding diversity associated with protein primary structure (amino-acid sequence and composition). However, the answers to many of these questions are less than satisfactory when addressing diversity due to carbohydrate structure.

Proteins produced using recombinant technology are frequently heterogeneous in their protein primary structure. Protein structural heterogeneity can include the presence of *N*-formyl methionine, truncated species due to internal initiation sites or proteolytic processing, and replacement

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of methionine by norleucine. For example, tissue plasminogen activator protein from CHO cells is produced as a mixture of one-chain and two-chain forms; recombinant GM-CSF from yeast is a mixture of two species with different *N*-termini due to proteolytic processing. For these examples, the number of different molecular species due to primary structure differences is limited and can be determined by standard methods of protein chemistry. In contrast is that heterogeneity due to the presence of carbohydrate. The oligosaccharide structures associated with a single site of glycosylation on erythropoietin include complex bi-antennary, tri-antennary, tetra-antennary, and tetra-antennary with *N*-acetyllactosaminyl repeats, in addition all species being variably sialylated. Therefore, one can easily appreciate the difficulty in completely characterizing a recombinant glycoprotein bearing one glycosylation site. The characterization becomes further complicated when multiple sites of glycosylation are indicated.

Defining the heterogeneity of a biological is necessary for evaluating consistency of the product. Each lot of a biological product is analyzed using appropriate analytical tests to measure product identity, purity and to demonstrate consistency in heterogeneity. Techniques are available that can quantitate primary protein structure heterogeneity in rapid and reproducible fashion. These tests are frequently incorporated into routine in-process monitoring which occurs during manufacturing.

Sugar-mapping procedures, analogous to peptide mapping, can quantitate proportions of glycoprotein species that differ in oligosaccharide structure and may be useful for demonstrating consistency between different production lots of a glycoprotein. However, current technology does not yet lend itself to routine and reproducible determination of oligosaccharide structures. Monosaccharide composition analysis can reveal the relative ratios of sugars to give an indirect determination of the oligosaccharides present. The site of attachment of oligosaccharides to a glycoprotein can be assessed by using glycosidases to cleave specific oligosaccharide chains coupled with analysis by acrylamide gel electrophoresis or peptide mapping, or by determining the ability of the glycoprotein to bind to different lectins. These procedures are qualitative at best, are not sensitive to heterogeneity in oligosaccharide structure, and cannot produce quantitative results appropriate for in-process or lot-release testing. Unfortunately, at present there is no simple procedure that can be used for the routine analysis of oligosaccharide structures located at specific sites on a glycoprotein.

Rapid progress is being made in oligosaccharide structure determination and separation and it is anticipated that within the near future, techniques for the routine analysis of oligosaccharide structure will be widely available. Given the availability of a rapid means for determining carbohydrate structure, a major challenge to the glycobiologist, manufacturers of clinically relevant glycoproteins, and regulatory agencies will be to determine how best to use this structural information in the development of safe and effective glycoproteins.

Clearly, the ability to determine oligosaccharide structure rapidly and reproducibly will contribute to the monitoring of the manufacturing process and result in improved product consistency. The production and processing of a recombinant glycoprotein is a very complex process involving the sequential action of enzymes on the protein as it is transferred through the golgi and intracellular compartment. "Stressing" the cell during fermentation by environmental changes such as glucose starvation or changes in intracellular pH may dramatically alter the presence and structure of oligosaccharides. Furthermore, protein purification methods frequently show potential for selecting protein subsets which consist of populations of proteins with the same protein primary structures and different oligosaccharide structures. Therefore, it is important to identify those fermentation parameters that can affect oligosaccharide structure and those purification steps capable of separating glycoprotein subsets. Appropriate tests and controls may then be implemented to ensure the consistent processing of the oligosaccharide, especially when the manufacturing process of a glycoprotein is modified.

The ability to define analytically the different species of a glycoprotein is just the first step in determining the biological significance of oligosaccharide structural diversity. The specific effect of oligosaccharide structure on the biological activities of a glycoprotein is still a relatively new area of investigation, but it is widely accepted that oligosaccharides can play a major role in determining the potency of a glycoprotein. For example, an important role for oligosaccharides has been demonstrated for many therapeutic glycoproteins: desialylation of erythropoietin is accompanied by a complete loss of *in vivo* activity; variable occupancy of glycosylation sites on GM-CSF has been correlated with differences in biological activity, clearance, and organ distribution; and occupancy of glycosylation sites on tissue plasminogen activator protein has been correlated with differences in clearance.

Oligosaccharides are important contributors to the clearance, biological specific activity, solubility, stability, antigenicity, and lectin binding properties of glycoproteins. Frequently, all of these properties are affected by the population of oligosaccharides present at specific sites on a glycoprotein. However, the effect of particular glycan structures at glycosylation sites on biological activity has been far more difficult to assess. Attempts to correlate antennary structure with biological activity for erythropoietin have not provided definitive insights concerning the specific interaction and importance of the complex antennary structure. *In vivo* biological activity of erythropoietin was greatest in preparations with a high ratio of tetra-antennary structure to bi-antennary structure. It is still not known whether the greater activity of the tetra-antennary species is due to decreased clearance of the protein, enhanced trafficking to

target tissue, or other undefined interaction. Determining the contribution of specific glycan structures to important physiological parameters will be a formidable task.

An important consideration in evaluating the role of carbohydrate is the specific test that is used to assess "biological activity". Many tests for biological activity determine the effects of the glycoprotein on cells in culture. However, the evaluation of these results is based on the assumption that the cell culture model accurately reflects the glycoproteins ability to elicit a clinically relevant effect. For example, deglycosylated hCG is unable to elicit detectable increases in cyclic AMP in rat Leydig cell preparations and can act as an antagonist to active, glycosylated hCG. However, deglycosylated hCG can elicit early testosterone responses in monkeys equivalent to that of intact hCG. In this case, evaluation of the clinical efficacy of a protein based solely on its ability to elicit a second messenger could be a misleading indicator of therapeutic effect.

Evaluation of biological activity based only on cell culture models or *in vitro* tests such as ligand binding may be misleading for other reasons. The *in vivo* biological activity of a glycoprotein will be related to its clearance, organ distribution, and metabolism. An extreme example is erythropoietin whose *in vitro* activity is enhanced upon removal of terminal sialic acids while such treatment results in a complete loss of *in vivo* activity. This has been attributed to rapid clearance of the desialylated protein from the circulation. The accepted *in vivo* test for erythropoietin, a mouse bioassay, is sensitive to the presence of sialic residues. However, many other assays that are utilized as measures of potency for biologists such as monoclonal antibodies, thrombolytics, and colony stimulating factors do not incorporate tests that would reflect the physiological requirement for the carbohydrate moiety. These considerations are not meant to discourage the development of appropriate *in vitro* assays to measure biological effects. However, too often one reads the premature conclusion, "Carbohydrate is not important for *in vivo* activity" when in fact there might be important pharmacological interactions occurring that are not being measured by the particular "*in vitro*" test.

The difficulty in obtaining a complete description of a protein's oligosaccharide structures is also complicated by the question of defining what structure is representative of the "naturally occurring protein". For example, human erythropoietin has been isolated from the urine of patients with aplastic anemia and used as the relevant human standard erythropoietin for structural analysis of the oligosaccharide chains. Human tissue plasminogen activator protein isolated from the supernatants of human Bowes melanoma cells has been used for the characterization of its oligosaccharide structures. For these and other proteins of therapeutic potential it may not be possible to isolate enough of the naturally occurring protein; i.e., that which occurs normally in humans, for detailed structural studies to define a standard. Glycoprotein hormones isolated from different individuals may also have different populations of oligosaccharide structures.

The inability to define a "natural" form need not be a critical issue hindering the use and evaluation of a glycoprotein. Each biological must be characterized separately regarding safety, potency, and efficacy regardless of how similar (or dissimilar) it may be to its native counterpart. It may be more appropriate to identify those oligosaccharide structures that are "foreign", determine the potential toxicity or adverse effects of such structures, and develop methods for the analytical determination of such structures in glycoproteins.

Glycoproteins exist as populations which differ in their carbohydrate structure. In a perfect world one could require each protein species to be purified and its activity determined. Realistically, assessment of the biological relevance of oligosaccharide structure is usually made by examining subsets of glycoforms prepared using relatively crude separations that include charge separations based on differences in sialic acid content, lectin separations of species that differ in antennary structure, or production separations that rely upon different host-cell systems to produce different populations of glycoform. A major contribution to our understanding of the role of carbohydrate would be the ability to efficiently produce glycoproteins with defined oligosaccharide structures.

It is recognized that glycoprotein diversity occurs naturally and may impart a functional benefit to the protein that is not readily discernible. Therefore, the heterogeneity of recombinant glycoproteins may actually provide a therapeutic benefit. Will the administration of a single glycoform produce a better therapeutic profile or a better vaccine than a mixture of glycoforms? The answer will be even more relevant to the development of future generations of therapeutic glycoproteins.

In summary there are a number of important questions that will occupy glycobiologists in the upcoming years. A major challenge is the development of methods for the routine analyses of carbohydrate structures that can be easily reproduced and verified in different laboratories. Of greater importance will be the effort necessary to demonstrate the effects of specific glycan structures on glycoprotein function.

Letters or comments relating to this article would be received with interest by Pauline Rudd, Assistant to the Special Advisory Editor, R. A. Dwek.