Receptors & Clinical Investigation 2015; 2: e758. doi: 10.14800/rci.758; © 2015 by John D. Chung, *et al*.
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RESEARCH HIGHLIGHT

Computational characterization of Afucosylation-Based IgG1 heterogeneity

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Received: April 01, 2015
Published online: May 19, 2015

IgG1s produced by cell culture are heterogeneous with respect to their afucosylated Fc glycan content. Since afucosylation content dramatically changes the nature of IgG1s, there exists a need for methods capable of dissecting the contributions of different afucosylated IgG1 forms to biological activity. Recently, Zhan and Chung applied classical ligand-receptor mathematical analysis to receptor binding data obtained from heterogeneous mixtures of afucosylated IgG1s in order to develop methods capable of performing such operations [1]. By explaining important experimental observations and extracting valuable biochemical property information embedded in the data, their model provides a convincing demonstration of the role that mechanistic mathematical modeling can play in characterizing heterogeneous mixtures of complex molecules. This review highlights important features of their mathematical analysis from a drug development perspective.

Keywords: afucosylation; heterogeneity; antibody; ligand-receptor; FcgammaR

To cite this article: John D. Chung, *et al*. Computational characterization of Afucosylation-Based IgG1 heterogeneity. Receptor Clin Invest 2015; 2: e758. doi: 10.14800/rci.758.

Introduction

Antibodies represent the largest class of biologics in the marketplace, in the clinic, and in drug development pipelines [2]. Years of experience working with therapeutic antibodies has advanced all areas of biologic research, including the basic and clinical sciences, process and manufacturing sciences, and analytical methods development. It is now known that antibody Fc-mediated functions are strongly influenced by the presence of a core fucose residue attached to the carbohydrate bound to the Asn297 glycosylation site [3-7]. This has resulted in glycoengineering efforts to optimize antibody Fc-mediated activity [8,9]. Experience gained in the process and manufacturing sciences has enabled worldwide antibody development efforts in developed and developing countries [10,11]. The need to adequately monitor the quality of therapeutic antibodies has resulted in the standardization of advanced technologies capable of characterizing glycoform heterogeneity [12]. More recently, these developments have enabled advances in the area of biomedical computing as applied to drug characterization [1].

Zhan and Chung recently demonstrated the ability of molecular-mechanistic ligand-receptor mathematical modeling to resolve experimental discrepancies that resulted from the use of empirical activity correlations and sample average metrics of protein quality [1,13]. Currently, the afucosylated Fc glycan fraction, denoted by p, is used to quantify IgG1 afucosylation content. Samples with high afucosylated Fc glycan fractions have been found to exhibit increased FcγRIIIa receptor binding activity, and vice-versa. Previous attempts to mathematically quantify this relationship

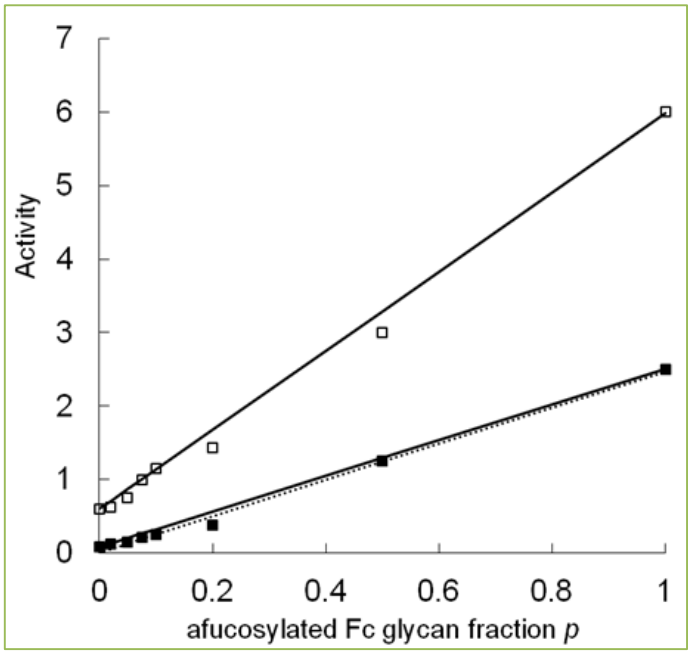

Figure 1. *Activity* as a function of afucosylated Fc glycan fraction *p*. The individual points were estimated from binding curves associated with different binary controlled mixtures of pure homogeneous fucosylated and afucosylated IgG1 (□= V158, ■=F158). The solid lines were generated using the equilibrium dissociation constants K_A and K_F. The dotted line represents a least-squares fit of the individual activity data points. Adapted from Zhan and Chung[1]. Reprinted with permission [1].

involved the use of empirical methods [13]. These exercises revealed that a unique activity-p correlation does not exist; the use of different samples resulted in different correlations. Due to the limitations of empirical methods, explanations for these differences could not be provided, thus limiting the reliable use of such relationships. Zhan and Chung were able to provide explanations for these observations by using well established mechanisms in biochemistry to develop a general mathematical framework applicable to analyzing a large class of heterogeneous ligand-receptor systems [1].

The fundamental issue associated with the analysis of the afucosylated IgG1 ligand-receptor system concerns the need to properly account for the different IgG1 forms that contribute to biological activity. In this regard, the afucosylated Fc glycan fraction metric p is of little assistance because it does not distinguish among the different IgG1 forms. An antibody contains two heavy chains, and each heavy chain has one potential Fc afucosylation site. Therefore, three antibody forms are possible, with each form differentiated by its afucosylation content: the homogeneous fucosylated IgG1, the hemi-afucosylated IgG1, and the homogeneous afucosylated IgG1. All three forms contribute to biological activity [1,14,15]. By directly addressing these considerations, Zhan and Chung dissected this system to extract valuable biochemical property information embedded in the data that had hitherto remained unrevealed.

Results and Discussion

Heterogeneous Ligand-Receptor Mathematical Modeling

To overcome the limitations associated with the use of empirical correlations and sample average metrics of IgG1 quality, Zhan and Chung applied classical competitive ligand-receptor mathematical modeling to the IgG1 Fc-FcγRIIIa ligand-receptor system to specifically account for the receptor binding activities of the three IgG1 forms that constitute a heterogeneous IgG1 sample [1]. The general mathematical activity-composition structure imposed on this ternary system by the competitive ligand-receptor mechanism was shown to be given by:

$$activity = \frac{X_F}{K_F} + \frac{X_{AF}}{K_{AF}} + \frac{X_A}{K_A} \qquad (1).$$

K_F, K_{AF} and K_A denote the equilibrium dissociation constants, while X_F, X_{AF} and X_A denote the mole fractions of the homogeneous fucosylated, hemi-afucosylated and homogeneous afucosylated IgG1 forms, respectively.

Equation (1) provides the means to formally account for the contributions of binding constant, $1/K_i$, and relative concentration differences of the different IgG1 forms to receptor binding activity. The ability of equation (1) to accurately predict heterogeneous sample receptor binding activity was demonstrated by Zhan and Chung using data published by the Roche organization, which was obtained from binary mixtures of pure homogeneous fucosylated and afucosylated IgG1 [13]. When the activity of the heterogeneous mixtures is computed using equation 1 and the pure component binding constants K_F and K_A, good agreement between the data is observed (Fig. 1).

Equation (1) highlights the fundamental danger associated with the overreliance on empirical activity correlations. Defining ternary system activity requires that two independent mole fractions in addition to the overall IgG1 concentration are specified. However, because many combinations of the mole fractions can lead to the same sample activity, activity cannot be used to define a unique set of compositions. With respect to afucosylation content, previous attempts to characterize this system using empirical correlations effectively amounted to assuming a homogeneous system, whereby activity is solely determined by a single independent variable and vice-versa. Although the use of the afucosylated Fc glycan metric p provides the impression that heterogeneity is adequately taken into account, attempts to correlate activity

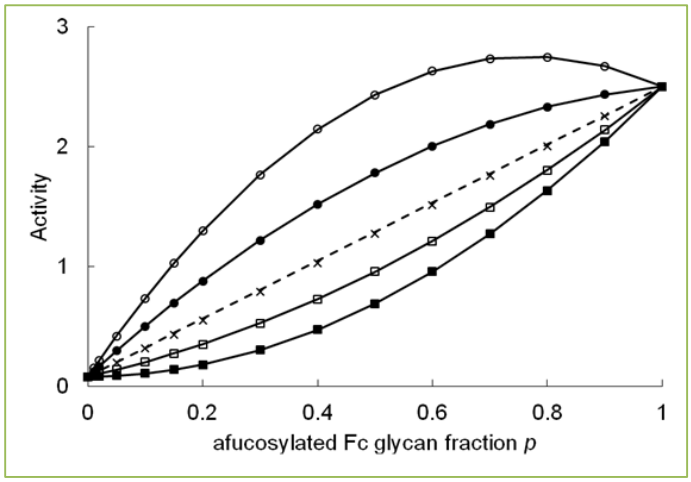

Figure 2. *Activity* as a function of afucosylated Fc glycan fraction *p* for random afucosylation. The curves were generated for different values of K_{AF} for the F158 FcγRIIIa polymorphism. The values of K_A and K_F are identical to the values used to generate figure 1. The dashed line (without symbols) represents the binary homogeneous activity-*p* curve shown in figure 1. Discrete data points from the locus, $K_{AF}=2K_A$, are denoted by X. The solid curves were computed for K_{AF} values of 0.7 K_A (○), 1.1 K_A (●), 4K_A (□) 0.9K_F (■). Adapted from Zhan and Chung [1]. Reprinted with permission [1].

directly with p implicitly assume that activity depends on a single independent variable. Note, however, that when the equilibrium constants of the different IgG1 forms are numerically equal, the different afucosylated IgG1 forms are no longer differentiated by their binding activities and the system becomes effectively homogeneous. Zhan and Chung showed that this scenario does not apply to the afucosylated IgG1 system [1].

Random Afucosylation

Zhan and Chung were able to show that under certain conditions, equation (1) could be simplified and that the number of independent variables needed to determine activity could be reduced to one [1]. The binary homogeneous system, characterized by a vanishing X_{AF} (Fig. 1), was one such example. However, Zhan and Chung provided a more powerful example that arises when the afucosylated Fc glycans are randomly distributed among the different IgG1 forms. Under this condition, they were able to show that equation (1) allows receptor binding activity to be expressed as a function of the single independent variable p (Fig. 2). Several features of the resulting activity-p curves differentiate them from the binary homogeneous system activity-p curve (Fig. 1). In general, activity will vary non-linearly with respect to p as a direct consequence of the presence of the hemi-afucosylated form [1]. Also, for certain values of K_{AF}, activity exhibits a maximum with respect to p so that a given activity may result from different values of p. Although activity is specified by the single independent variable p, the activity curves retain features of the multivariable system. An interesting special case arises when $K_{AF}/K_A = 2$. Under these circumstances, the ternary or "…biosimilar system curve converges to the linear activity-p curve of the binary homogeneous antibody system…" [1] (Fig. 1), and the afucosylated Fc glycan fraction is the sole determinant of activity, irrespective of how the afucosylated Fc glycans are distributed among the IgG1 backbones. Since different activity-p correlations are obtained from different samples, this special case does not apply [13].

Zhan and Chung were able to develop a method for computing the value of K_{AF} using receptor binding activity data obtained from production samples with low p. The non-linear activity curves shown in figure (2) were accurately approximated by a family of linear relationships with slopes directly related to the value of K_{AF} [1]. Zhan and Chung applied this relationship to analyze receptor binding data to compute $K_{AF}/K_A \approx 0.6$, revealing that on a molar basis the hemi-afucosylated IgG1 is the most active IgG1 form. On an afucosylated Fc glycan basis, the relative activity of the hemi-afucosylated form is even greater. Although a linear relationship between receptor binding activity and p has been previously observed for low p samples, the empirical nature of the results did not permit further data analysis [13]. Since many therapeutic antibodies, including Rituximab and Palivizumab, are characterized by low p [6,13,16], the linear activity-p relationship developed by Zhan and Chung can be applied to characterize a variety of antibodies, including currently-marketed, follow-on, and new drugs. Although the computational method described by Zhan and Chung is restricted to low p samples, once the equilibrium constants have been determined, equation (1) and figure 2 can be used to analyze receptor binding data for samples characterized by any p value, encompassing such products as Traszutumab, with p~0.25 [17], and possibly the recently approved Obinutuzumab. Although p values for Obinutuzumab do not appear to be in the public domain, bisecting GlcNac technology has been reported to result in high, but not complete, afucosylation [8,18].

Biochemical Data Integration and Data Quality

The mathematical structure revealed by the analysis of Zhan and Chung in equation (1) provides a means to integrate data obtained from different sources and to insure data quality and consistency. The need to resolve heterogeneous products along multiple composition axes in addition to concentration has the potential to give rise to unprecedented information and data. Data on the identities, concentrations and specific potencies of the dominant biologically active forms that constitute a sample is essential for proper product characterization. However, because such data can, in

principle, be assembled using a variety of methods and techniques, a means to insure data quality and relevance must be available. This is an important consideration because *ex vivo* biochemical property information has little intrinsic meaning. Its value is derived from its participation in an *ex vivo* biological system as an integral part of a whole. At a minimum, such information must be able to be integrated so as to reconstruct *ex vivo* system properties. Equation (1) provides a means to perform this operation. Deviations from this relationship that result during the data integration process are indicative of any of three major undesirable situations: 1) erroneous and/or inconsistent data, 2) unrevealed sources of significant biological activity and 3) unrevealed mechanisms of interaction. Since equation (1) does not depend on any specific assay format, the consistency and quality of the available information can be assessed regardless of whether the information can be theoretically related to data acquired by different means and from different *ex vivo* systems.

Computational Characterization of Heterogeneous Biologics

Advances in science and technology in the drug development arena have motivated the need for more sophisticated methods to characterize heterogeneous mixtures of complex molecules. The use of mechanistic mathematical models to meet these needs represents a natural step in the evolution of applied computational biology. Empirical characterization techniques have proven themselves to be indispensable for studying new phenomena, and they will continue to play vital roles in the discovery process. However, when a field matures, such methods are incapable of processing and integrating the volume of information that accompanies increased scientific knowledge. Molecular mechanistic mathematical methods provide a means to overcome this limitation. The work of Zhan and Chung demonstrates that such a transition is already underway for heterogeneous afucosylated IgG1s. However, because nearly every biologic in the marketplace is governed by the competitive ligand-receptor binding mechanism and thus the mathematical structure imposed by equation (1), it is only natural to ask whether all biologics should be analyzed in an equivalent manner. The heterogeneity associated with IgG1 afucosylation is tractable, involving only three dominant forms that are clearly differentiated by their biological activities. With systems that involve more than three components and have poorly-characterized structure-activity relationships, the theoretical framework highlighted provides a foundation upon which to begin such an exercise.

The Quality by Design (QbD) initiative and the *totality of evidence* philosophy of the Food and Drug Administration highlight the important roles that product quality and adequate product characterization play in the licensing process. The need to ascertain and target a product quality level early in the drug development process will require that the impact of cell lines, expression systems and processes on product quality be well documented. However, because final product quality can never be precisely predicted *a priori*, product characterization methods capable of providing information on the identities, concentrations and potencies of the relevant biological forms are crucial. The computational methods highlighted directly address this need and may prove to be indispensable, particularly when direct experimental approaches are either unavailable or impractical. It is important to emphasize that the methods developed by Zhan and Chung are based on the straightforward mathematical consequences of well-established and well-accepted, decades-old mechanisms in biochemistry and receptor biology. The data obtained from using these methods are as meaningful as the data from which they are derived. Therefore, the use of molecular mechanistic-based mathematical models as a foundation for the computational characterization of biologics should be of interest to all parties engaged in biologics development.

Conflicting interests

The authors have declared that no competing interests exist.

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