Identification of a Guinea Pig Fcγ Receptor that Exhibits Enhanced Binding to Afucosylated Human and Mouse IgG

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Abstract
Glyco-engineered recombinant antibodies are currently being developed as the next generation therapeutics to treat various human diseases, including cancer, autoimmunity and infection. Antibodies lacking core fucosylation show great increase in affinity for FcγRIIIA, leading to an improved receptor-mediated effector function. While afucosyl human IgG1 exhibits 50-100-fold increase in antibody-mediated cellular cytotoxicity (ADCC), a key immune effector mechanism underlying the anti-cancer effect of some approved therapeutic antibodies, it is not clear whether such glyco-engineered antibodies would find similar use for infectious disease. Due to the species difference, human antibodies may have different binding properties towards corresponding IgG receptors from animals used for modeling infection and intoxication. During the course of studying a recombinant human IgG1 in neutralizing diphtheria toxin (DT) in Guinea pigs (Cavia porcellus), we identified a previously uncharacterized Guinea pig protein H0VDZ8 from UNIPROT database that shows high sequence homologies to human FcγRIIIA and mouse FcγRIV. This Fcγ receptor, which we named as gpFcγRIV, also demonstrates functional similarity although not to the same extent as the human and mouse counterparts, in that it binds to afucosyl human and mouse IgG much stronger than to the wild type antibodies. Thus, Guinea pigs can be used to compare the efficacies of wild type vs. afucosyl anti-DT human IgG1 in toxin removal and animal protection. Molecular and functional characterization of human FcγRIIIA and mouse FcγRIV equivalents in other species could expand the list of preclinical animal models for testing afucosyl human antibodies in treating various human diseases.

Keywords: Immunoglobulin G; Fc receptor; Afucosylation; Guinea pig

Abbreviations
CHO: Chinese Hamster Ovary; ADCC: Antibody-dependent Cellular Cytotoxicity; ADCP: Antibody-dependent Cellular Phagocytosis; Fut8: Alpha-(1,6)-fucosyltransferase 8; TALEN: Transcription Activator-like Effector Nuclease

Introduction
Monoclonal antibodies (Mabs) are being developed as therapeutics for treating various human diseases. The vast majority of marketed IgG Mabs are produced in mammalian cells, especially Chinese hamster ovary (CHO) cells. IgG1 antibodies produced from wild type CHO cells have a fucose residue in a 1,6 linkage to the first GlcNAc of the oligosaccharide core (“core fucosylation”) in their bi-antenna glycan attached to asparagine (Asn) residue 297 in the Fc region. It has been well established by many laboratories that the addition of core fucosylates diminishes the affinity of IgG Fc to the human FcγRIIIA receptor expressed on natural killer cells, macrophages, neutrophils, and other immune cells. As a result, IgG molecules lacking a core fucose residue bind with 50-fold greater cellular immune functions, for example, antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) [1].

Currently, afucosyl antibodies with enhanced ADCC towards target cells are mainly being pursued in the immuno-oncology area. For example, Mogamulizumab is an afucosyl humanized Mab targeting CC chemokine receptor 4 (CCR4), approved in Japan in 2012 for the treatment of relapsed or refractory adult T-cell leukemia-lymphoma (ATL) [2]. Obinutuzumab against CD20 is the second afucosyl Mab approved by US FDA in 2013 for treating B cell lymphoma [3]. Besides these two approved, more afucosyl antibodies are in the research pipeline, and the pharmaceutical industry has recognized the advantages of fucose-free therapeutic antibodies [3]. While the benefits of afucosyl Mabs in cancer immunotherapy are obvious, so far there are only two reports in infectious disease area supporting their potential application, where afucosyl antiviral antibodies show enhanced cell-mediated antiviral potency against the HIV and Ebola viruses [4,5].

The main reason for this deficiency is due to the lack of knowledge on the binding properties of human IgGs with or without fucose towards corresponding antibody receptors from animals used for modeling infection and intoxication. Among the human Fcγ receptors, only FcγRIII is sensitive to IgG defucosylation. In mice, mFcγRIV is regarded as the functional homologue of hFcγRIIIA. Accordingly, only mFcγRIV is sensitive to IgG defucosylation among all the murine Fcγ receptors [6]. Little is known on the counterparts of hFcγRIIIA and mFcγRIV in other species, as well as their binding sensitivity towards afucosyl antibodies.

During the course of studying a human anti-diphtheria toxin IgG1 Mab in Guinea pigs (Cavia porcellus), we first asked: 1) whether hlgG1 can bind to Guinea pig FcγR, and 2) whether there is an equivalent of hFcγRIIIA and mFcγRIV in Guinea pigs which exhibits enhanced binding to afucosyl hlgG1. Our studies show that a previously uncharacterized Guinea pig protein H0VDZ8 from UNIPROT database is a structural and functional homologue to human FcγRIIIA and mouse FcγRIV.

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Materials and Methods

Cell line generation: CHO cell lines expressing the functional hFcRRIIIA, mFcγRIV or gpFcγRIV complexes on the cell surface were generated with the "Toggle-In" CHO system (Antagen Pharmaceuticals, Inc., Boston, MA). The establishment of the anchor "Toggle-In" CHO cell line and the construction of pTOG3 and pTOG4 expression vectors with alternate use of Hygromycin B and Puromycin as selection markers will be published in more detail elsewhere. Briefly, an anchor "Toggle-In" CHO cell line was selected by FACS sorting on d1EGFP expression at a CHO genomic "hot-spot". Several genes involved in enhancing protein expression, e.g., SRP54, SRP9, SRP14, ERO1-L and FGF9 were sequentially integrated at this "hot-spot" by replacing d1EGFP with Cre-LoxP recombination-mediated cassette exchange (RMCE). A CHO cell line thus generated is named as the master "Toggle-In" line, which has been used for establishing other cell line models. Herein for example, human FcR common γ chain in pTOG4 was first transfected into the master "Toggle-In" line, and selected with Puromycin (10 μg/mL). An FcRγ-positive clone was picked and further transfected with hFcRRIIIA-V158, mFcγRIV or gpFcγRIV, all cloned in pTOG3, respectively, and selected with Hygromycin B (1 mg/mL). Single CHO clones of double transfectants were screened and confirmed by FACS staining with hIgG1 or mIgG2a.

Expression of wild type and afucosyl antibodies: A ScFv-hlgG1 construct, where its ScFv part utilizes anti-TNFα antibody sequence to fuse with human IgG1 Fc, was cloned into pDirect4.0 expression vector (Antagen), and transfected into either wild type or proprietary Fut8-/- CHO cells (Antagen). This Fut8-/- CHO cell line was established in-house with TALEN technology. Detailed method and genomic sequences of the two TALEN-targeted Fut8 alleles will be disclosed elsewhere. A mouse hybridoma secreting IgG2a antibody (PK136, ATCC, Manassas, Virginia) against mouse NK1.1 was transfected with pTOG4, in-house with TALEN technology. As FcR common γ chain co-expression is required for proper protein folding and cell surface display of hFcRRIIIA or mFcγRIV, we took the advantage of our "Toggle-In" system (Antagen), where Cre-LoxP based sequential integration of exogenous genes into the same genomic locus is exploited for isogeneic co-expression of the hFcRRIIIA-hFcRγ, mFcγRIV+hFcRγ or gpFcγRIV+hFcRγ complex (See Methods).

When titrated amounts of wild type and afucosyl hlgG1 were added to hFcRRIIIA-V158 expressing CHO cells, afucosyl hIgG1 demonstrated much better binding than wild type hIgG1. The EC50 of afucosyl hIgG1 binding to hFcγRIIIA-V158 expressing CHO cells, afucosyl hIgG1 demonstrated much better binding than wild type hIgG1. The EC50 of afucosyl hIgG1 binding to hFcγRIIIA-V158 expressing CHO cells was >2000 ng/mL (12.5 nM) (Figure 2). We estimate that by our method, there is >10-20-fold increase in hIgG1 binding to

Results and Discussion

To search for Guinea pig homologue(s) of hFcRRIIIA and mFcγRIV, we blasted UNIPROT database and found a few candidate Guinea pig proteins that show various homologies. One uncharacterized protein H0VDZ8 has not been assigned a gene name shows the highest homologies: 55.3% identical and 72.5% similar amino acids with hFcγRIIIA; 54.9% identical and 71.4% similar amino acids with mFcγRIV (Figure 1). H0VDZ8 could be the potential Guinea pig equivalent of hFcγRIIIA and mFcγRIV.

Based on its amino acid sequence, we ordered full gene synthesis for H0VDZ8 as "gpFcγRIV" with CHO codon optimization (Integrated DNA Technologies, Inc., Coralville, Iowa), and cloned it into the pTOG3 vector (Antagen). As FcR common γ chain co-expression is required for proper protein folding and cell surface display of hFcRRIIIA or mFcγRIV, we took the advantage of our "Toggle-In" system (Antagen), where Cre-LoxP based sequential integration of exogenous genes into the same genomic locus is exploited for isogeneic co-expression of the hFcRRIIIA-hFcRγ, mFcγRIV+hFcRγ or gpFcγRIV+hFcRγ complex (See Methods).

When titrated amounts of wild type and afucosyl hlgG1 were added to hFcRRIIIA-V158 expressing CHO cells, afucosyl hlgG1 demonstrated much better binding than wild type hlgG1. The EC50 of afucosyl hlgG1 binding to hFcRRIIIA-V158 is about 200 ng/mL (1.25 nM), whereas that of the wild type hlgG1 is >2000 ng/mL (12.5 nM) (Figure 2). We estimate that by our method, there is >10-20-fold increase in hlgG1 binding to

**Figure 1:** Alignment of Guinea pig protein H0VDZ8 with hFcγRIIIA (P08637, upper) or mFcγRIV (Q8R477, lower).
Got very similar results: The EC50 of afucosyl hIgG1 binding to gpFcγRIIIA and Fut8-/- CHO cells. Could be an alternative to cloning the VH VL genes from the hybridoma antibodies if they are of ADCC-enabling IgG2a/2b/2c isotypes. This compare hybridoma-derived wild type and afucosyl parental mouse antibodies. The advantage of CRISPR knockout of mouse Fut8 gene in hybridoma, and isotype (H0VDZ8) in mouse models of disease, researchers can directly take the therapeutic values of afucosylated humanized murine antibody (hIgG1 defucosylation). In other words, to demonstrate the enhanced murine immune system could largely recapitulate the benefits of hIgG1 in binding to gpFcγRIIIA (Figure 2). This suggests that murine immune system can largely recapitulate the benefits of hIgG1 defucosylation. In other words, to demonstrate the enhanced therapeutic values of afucosylated humanized murine antibody (hIgG1) isotype in mouse models of disease, researchers can directly take the advantage of CRISPR knockout of mouse Fut8 gene in hybridoma, and compare hybridoma-derived wild type and afucosyl parental mouse antibodies if they are of ADCC-enabling IgG2a/2b/2c isotypes. This could be an alternative to cloning the VH VL genes from the hybridoma and expressing the chimeric antibody genes with hlgG1 Fc in wild type and Fut8/- CHO cells.

However, the binding of hlgG1 to gpFcRγRIV does not show much enhancement after defucosylation (Figure 2). We repeated the assay and got very similar results: The EC50 of afucosyl hlgG1 binding to gpFcRγRIV is about 289.3 ng/mL (1.81 nM), whereas that of the wild type hlgG1 is 712.1 ng/mL (4.45 nM) (data not shown), a mere 2.5 fold enhancement. For mlgG2a, although it does not bind to hFcγRIIIA-V158, its binding to gpFcRγRIV is dramatically enhanced after defucosylation (Figure 2).

Our studies answered the initial questions that hlgG1 can bind to Guinea pig FcγR and H0VDZ8 is a structural and functional homologue to human FcγRIIIA and mouse FcγRIV. In fact, based on the mean fluorescence intensity (MFI) data in flow cytometry, the binding affinity of hlgG1 to this gpFcγRIV is about 100-fold and 10-fold over the binding affinity of hlgG1 to FcγRIIIA and mFcγRIV, respectively. We believe these are not due to the cell line differences in receptor surface expression because of integration site and copy number variation seen in the conventional transfection method, as our "Toggle-In" system can eliminate such factors by site-specific isogeneic expression of exogenous genes with equal copy number. Perhaps also due to this stronger base line binding of hlgG1 to gpFcγRIV, the difference between the wild type and the afucosyl forms of hlgG1 in binding to gpFcγRIV is not as dramatic. Therefore, the benefit of afucosyl hlgG1 in Guinea pig model could be under-manifested as in humans. From proof-of-concept point of view, it remains to be tested whether murinized human therapeutic IgG with mlgG2a/b/c Fc tail would better exemplify the benefits of afucosyl antibodies in disease models where Guinea pigs are used as host.

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References
1. Listinsky JJ, Siegal GP, Listinsky CM (2013) Glycoengineering in cancer therapeutics: a review with fucose-depleted trastuzumab as the model. Anticancer Drugs 24: 219-227.
2. Beck A, Reichert JM (2012) Marketing approval of mogamulizumab: a triumph for glyco-engineering. MAbs 4: 419-425.
3. Small S (2013) Approval of obinutuzumab as a breakthrough therapy for chronic lymphocytic leukemia. Clin Adv Hematol Oncol 11: 809-810.
4. Forthal DN, Gach JS, Landucci G, Jez J, Strasser R, et al. (2010) Fc-glycosylation influences Fcy receptor binding and cell-mediated anti-HIV activity of monoclonal antibody 2G12. J Immunol 185: 6876–6882.
5. Zeitzl L, Pettitt J, Scully C, Bohorova N, Kim D, et al. (2011) Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. Proc Natl Acad Sci USA 108: 20690–20694.
6. Nimmerjahn F, Ravetch JV (2005) Divergent immunoglobulin G subclass activity through selective Fc receptor binding. Science 310: 1510-1512.