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Human Fc receptor-like 5 distinguishes IgG2 disulfide isoforms and deamidated charge variants

Oyeleye Alabi[1], Jessica Dement-Brown, Mate Tolnay[*]

Office of Biotechnology Products, Center for Drug Evaluation and Research, Food and Drug Administration, 10903 New Hampshire Ave., Silver Spring, MD 20993, USA

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ABSTRACT

Human Fc receptor-like 5 (FCRL5) is a novel IgG receptor. We reported that IgG2 samples display a thousand-fold range affinity for FCRL5, indicating that attributes beyond the isotype affect binding. We hypothesized that the complex interaction could be exploited to identify distinct changes in the IgG2 molecule. We investigated using surface plasmon resonance two factors that might affect the interaction between IgG2 and FCRL5; heterogeneity related to disulfide isoforms and charge variants. We found that panitumumab and denosumab samples enriched for the more flexible A disulfide isoform bound FCRL5 with two-fold and 82-fold higher apparent affinity, respectively, than the B isoform. We next assessed whether FCRL5 binding can distinguish panitumumab charge variants which increase during storage, using two approaches. First, samples were stored at 40 °C to promote acidic variants. Heat stressed panitumumab had up to four-fold higher apparent affinity for FCRL5. Next, we used conditions that promoted deamidation, a common cause of acidic variants. We found that deamidated panitumumab had up to 14-fold higher apparent affinity for FCRL5, indicating that deamidation promotes the interaction. Statistical analyses of kinetic parameters and similarity scores obtained from sensogram comparisons indicated that IgG2 disulfide isoforms, heat stressed and deamidated samples each bind FCRL5 differently. We conclude that based on FCRL5 binding, we can discern distinct changes in the IgG2 molecule, including the disulfide isoform structure and charge variants related to deamidation. Since both IgG2 deamidation and conversion of disulfide isoforms occur *in vivo*, these findings elucidate the biological FCRL5 ligand.

1. Introduction

The IgG2 subclass as a therapeutic agent is preferred in clinical settings where engagement of Fcγ-receptors and complement is not desired. There are currently four licensed therapeutic IgG2; panitumumab, denosumab, evolocumab and brodalumab. The IgG2 hinge contains four cysteine residues per heavy chain (H chain) and is able to form distinct disulfide bond structures, including dimers and isoforms, through inter- and intra-molecular rearrangements (Yoo et al., 2003; Wypych et al., 2008). Covalent dimers held together by two inter-molecular disulfide bonds have increased valency, which could play a role in efficient binding of bacterial carbohydrate epitopes (Yoo et al., 2003). Three intra-molecular IgG2 disulfide structures can also be formed. Four disulfide bonds are formed between the H chains in the A isoform, which is the classical structure. In contrast, the B isoform contains two disulfide bonds between the H chains, and the remaining cysteines in the upper hinge engage the Fab arms, resulting in a sterically restricted structure. The third isoform is a hybrid A/B structure. The *in vitro* antigen binding activity of some IgG2 was found to be affected by the disulfide isoform structure; the more flexible A isoform is reported as more potent (Dillon et al., 2008). The restricted hinge flexibility of the B isoform would also be expected to limit bivalent engagement of bacteria displaying rigid epitope distribution; however, we are not aware of any published report. Antibody producing cells were reported to contain and secrete mostly the A disulfide isoform, which is then slowly converted *in vivo* to the B isoform via the A/B hybrid (Liu et al., 2008).

The most common modification occurring during IgG storage is changes to the charge profile due to multiple chemical reactions, including asparagine deamidation, aspartate isomerization, C-terminal lysine cleavage, and N-terminal glutamatek cyclization (Khawli et al., 2010; Liu et al., 2011; Tang et al., 2013; Du et al., 2012). Non-enzymatic deamidation of asparagine residues and isomerization of aspartate are major IgG degradation pathways that proceed through a

Abbreviations: FCRL5, Fc receptor-like 5; H chain, heavy chain; SPR, surface plasmon resonance; RU, relative unit

* Corresponding author.
E-mail addresses: oyeleye.alabi@otsuka-us.com (O. Alabi), jessica.dementbrown@fda.hhs.gov (J. Dement-Brown), mate.tolnay@fda.hhs.gov (M. Tolnay).
[1] Present address: Otsuka Pharmaceutical Development and Commercialization, Inc., 2440 Research Boulevard, Rockville, MD 20850, USA.

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common succinimide intermediate. Asparagine deamidation rates depend on the primary sequence, higher order structure, solution pH and temperature (Pace et al., 2013; Zhang et al., 2014; Chelius et al., 2005; Robinson and Robinson, 2001a). Four deamidation sites were identified in the human IgG conserved region when stored under physiological pH (Chelius et al., 2005), of which only one asparagine deamidated at a significant rate (Liu et al., 2009). Importantly, the deamidation rates of two IgG2 and one IgG1 mAb were found comparable *in vivo* and *in vitro* (Liu et al., 2009). Deamidation was proposed to constitute a molecular clock reflecting protein age, and was predicted to have biological functions (Robinson and Robinson, 2001b).

FCRL5 is a novel IgG receptor with limited homology to Fcγ-receptors (Wilson et al., 2012; Franco et al., 2013). FCRL5 is expressed on plasma cells and a subset of mature B cells, with the capacity to modulate B cell antigen receptor signaling (Davis, 2007; Haga et al., 2007; Li et al., 2016). While Fcγ-receptors interact with the Fc portion, we proposed FCRL5 binds IgG via a two-step mechanism; first engaging the Fc portion and subsequently the $F(ab')_2$ portion (Franco et al., 2013). We proposed that the flexibility of the IgG hinge is required for proper alignment of the two interacting IgG-FCRL5 interfaces. Our model of FCRL5 interaction is based on experimental data using IgG1; its validity for IgG2 is yet to be established. We reported that seven monoclonal IgG2 displayed affinities from approximately 2 nM to 200 μM for FCRL5, as measured by surface plasmon resonance (SPR) (Franco et al., 2013). The variable binding of IgG2 remains unexplained and is examined in the present study. Two well-characterized, licensed human therapeutic IgG2, panitumumab and denosumab, were investigated for molecular attributes that might affect FCRL5 binding. Panitumumab and denosumab bind FCRL5 differently. Denosumab has lower affinity, binds with a rapid kinetics, and the interaction lacks the secondary binding component attributed to the $F(ab')_2$ portion. In contrast, panitumumab binds with higher affinity via a two-step mechanism (Franco et al., 2013).

In the present study, we assessed whether the IgG2 isoform structure or charge profile affects FCRL5 binding. We generated and assessed IgG2 representative of stress stability samples, which correspond to biologically relevant product variants.

2. Materials and methods

2.1. Reagents

Panitumumab (Vectibix) and denosumab (Prolia) were obtained from the National Institutes of Health's pharmacy (Bethesda, MD). Recombinant human FCRL5-His and anti-His mouse IgG1 were from R & D Systems. The following SPR reagents were from GE Healthcare; CM5 sensor chip, amine coupling kit, 10 mM pH 1.5 glycine buffer, 10 mM pH 5.5 sodium acetate, and nonspecific binding reducer. Cysteine, cystamine and guanidinium chloride were from Sigma-Aldrich. Acetonitrile, isopropyl alcohol, trifluoroacetic acid and tween 20 were from Fisher Chemicals. Sodium chloride and sodium acetate were from Mallinckrodt. PBS, HEPES and Tris were from Quality Biologicals. All chemicals and reagents were of analytical or molecular biology grade.

2.2. SPR analysis and calculation of similarity scores

Experiments were performed on Biacore T200 (GE Healthcare) as described, with modifications (Franco et al., 2013). The negative charge of the sensor surface was reduced by a blank (no protein) immobilization cycle using the amine coupling kit. Approximately 10,000 RU anti-His mouse IgG1 mAb was then immobilized on CM5 sensors using the amine coupling kit. Recombinant human FCRL5 containing the entire extracellular region with a C-terminal His-tag from a 4 μg/ml solution was captured on the anti-His surface. IgG2 samples were injected over FCRL5 at six panitumumab or eight denosumab concentrations (one of which was run in duplicate), two-fold serially diluted in HBS-P buffer containing 1 mg/ml nonspecific binding reducer. Association was allowed for 8 min at 20 μl/min at 25 °C, then dissociation was monitored for 10 min. Bound Ig and FCRL5-His were removed with two 1 min injections of 10 mM glycine-HCl, pH 1.5. Data were analyzed using Biacore T200 Evaluation software 3.0, subtracting the reference surface (immobilized anti-His mAb, but no captured FCRL5-His) and buffer control signals from each curve. Data were collected at a rate of 1 Hz and globally fitted by simultaneous numerical integration to the association and dissociation parts of the interaction, using the 1:1 or two-state kinetic analysis models. The apparent stoichiometry of the interaction was calculated according to the following formula: IgG2/FCRL5 = (Rmax of bound IgG2/FCRL5 on sensor) x 100/150, to consider the molecular masses of IgG2 (150 kDa) and FCRL5-His (100 kDa). Similarity scores were calculated using a novel feature of the Evaluation software 3.0. Data were normalized with respect to response, to reduce differences among experiments due to FCRL5 capture levels. Separate similarity scores were obtained for the entire interaction, the association phase and the dissociation phase, using a comparison window width of 3 SD, as published (Karlsson et al., 2016).

2.3. Generation of heat stressed and deamidation samples

For heat stressed samples, panitumumab was kept in the original formulation buffer (100 mM NaCl, 83 mM Na-acetate, pH 5.6-6.0) at 40 °C for two or four weeks under sterile conditions. To promote deamidation, panitumumab samples were exchanged into 100 mM phosphate buffer pH 7.4 and pH 8.0, containing 0.1% Na-azide, and kept at 37 °C for two or four weeks.

2.4. Redox enrichment of IgG2 disulfide isoforms

Disulfide isoforms A and B were enriched as published (Dillon et al., 2008). Briefly, to generate IgG2 enriched in disulfide isoforms A, the mAb at 5 mg/ml was exchanged into 0.9 M guanidine-HCl, 6 mM cysteine, 1 mM cystamine in 200 mM Tris, pH 8.0. To generate IgG2 enriched in disulfide isoform B, the mAb was exchanged into 6 mM cysteine, 1 mM cystamine in 200 mM Tris, pH 8.0. Samples were incubated at 2–4 °C for 48 h.

2.5. Reversed phase chromatography to separate disulfide isoforms

Samples were prepared at 1 mg/ml concentration in PBS buffer. Chromatographic conditions were as described (Wang et al., 2010). The AdvanceBio RP-mAb C8 column (3.5 μm, 450 Å, 50 mm × 2.1 mm) was from Agilent Technologies. An Agilent 1200 HPLC system equipped with a binary pump, auto sampler and UV detector was used. Mobile phase A contained 2% IPA and 0.1% TFA in water, while mobile phase B contained 70% IPA, 20% acetonitrile, 10% H_2O and 0.1% TFA. The separation gradient was 25–40% B for 30 min, with a flow rate of 0.5 ml/min. The column temperature was set to 85 °C and UV detection was carried out at 214 nm. Origin 8.0 software from OriginLab was used to quantify relative peak areas.

2.6. Cation exchange chromatography to separate charge variants

Weak cation exchange separation of samples was achieved using a Dionex Propac WCX-10 column (Agilent Technologies) and an Agilent 1200 HPLC system monitored at 280 nm, as described (Chu et al., 2007). Samples were run at room temperature at a flow rate of 0.5 ml/min. The column was equilibrated with 10 mM sodium acetate, pH 5.0 for 10 min and eluted with a mobile phase gradient of 0–100 mM NaCl in 10 mM sodium acetate, pH 5.0 for 120 min. Origin 8.0 software was used to quantify relative peak areas.