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Technical Note

Development of a label-free FcRn-mediated transcytosis assay for *in vitro* characterization of FcRn interactions with therapeutic antibodies and Fc-fusion proteins

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ABSTRACT

The neonatal Fc receptor (FcRn) binds to the Fc domain of IgG in a pH-dependent manner, guides the intracellular movement of the bound antibodies and protects them from lysosomal degradation. Proper characterization of Fc-FcRn interactions is fundamental to successful design, development, and production of Fc-containing therapeutic proteins because of the potential impact of such interactions on their *in vivo* pharmacokinetic behaviors. Here, we describe the development and characterization of a cell-based, label-free FcRn-mediated transcytosis assay that provides a functional readout to reflect the totality of Fc-FcRn interactions, including pH-dependent association and dissociation, as well as the intracellular trafficking of Fc-containing molecules in complex with FcRn. Our study demonstrates that this transcytosis assay can be used to evaluate FcRn binding of therapeutic antibodies and Fc-fusion proteins, including wild-type and engineered Fc variants with varying FcRn binding affinities, as well as oxidized and aggregated antibody samples. These results support the utility of an FcRn-dependent transcytosis assay for evaluation of both Fc-FcRn interactions and the structural integrity of Fc-containing therapeutic proteins pertinent to their pharmacokinetic behavior *in vivo*.

1. Introduction

The major histocompatibility complex (MHC) class I-related neonatal Fc receptor (FcRn) plays a central role in protecting IgG from degradation, thereby prolonging its half-life in circulation (Ward et al., 2015; Pyzik et al., 2015). Proper characterization of Fc-FcRn interactions is fundamental to the successful design, development and production of therapeutic IgG antibodies and Fc-fusion proteins due to the potential impact of such interactions on the *in vivo* pharmacokinetic (PK) profile.

FcRn is a heterodimer composed of a transmembrane heavy chain homologous to class-I MHC molecules and a soluble light chain, β2 microglobulin (B2M). FcRn binds to the Fc region of IgG in a pH-dependent manner such that only in an acidic environment (pH < 6.5) can the FcRn bind to IgG with high affinity, whereas it does not bind to IgG at physiologic pH or higher (> 7.4). This pH-dependent catch and release process allows FcRn to transport IgGs *via* two intracellular trafficking pathways. FcRn binds to the Fc region of IgG in acidic endosomes and protects IgG from lysosomal degradation by diverting them to a recycling pathway that returns the IgGs to the circulation. Additionally, the FcRn transports IgGs across various cell barriers *via* a transcytosis pathway to fulfill physiological needs such as transplacental delivery of maternal antibody to the fetus and transepithelial delivery of IgGs to mucosal sites (Ward et al., 2015). Modifications of the interactions between IgG and FcRn *via* genetic engineering have been shown to impact the half-life of therapeutic antibodies and Fc-fusion proteins (Ward et al., 2015; Pyzik et al., 2015). Thus, it is important to characterize Fc-FcRn interactions of Fc-containing therapeutic proteins to ensure that their Fc domains are functional and interact properly with FcRn.

A wide variety of *in vitro* assays have been developed to characterize IgG Fc-FcRn interactions, including cell-based and non-cell-based

Abbreviations: mAb, monoclonal antibody; IgG, immunoglobulin G; FcRn, neonatal Fc receptor; MHC, major histocompatibility complex; B2M, β2 microglobulin; TEER, trans-epithelial electrical resistance; MDCK, Madin-Darby Canine Kidney; FACS, florescence-activated cell sorting

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formats. Non-cell-based assays employing surface plasmon resonance (SPR) (Tesar et al., 2006), Bio-Layer Interferometry (Souders et al., 2015) and affinity chromatography (Schlothauer et al., 2013) have been commonly used to study interactions between Fc-containing proteins and FcRn. These methods measure the binding activity of Fc-containing proteins with soluble recombinant FcRn, either in solution or on an artificial solid surface under either acidic or neutral pH. On the other hand, cell-based assays utilize engineered cell lines expressing FcRn on the cell surface. Thus, binding of test molecules to membrane-bound FcRn is measured using either flow cytometry, ELISA, or Meso Scale Discovery (MSD) technologies (Mathur et al., 2013). Given that the interactions between IgG-Fc and FcRn involve association at acidic pH and dissociation at physiologic pH, as well as trafficking as a complex inside the cells, none of the previously reported FcRn binding methods is able to measure the combined effects of different aspects of Fc-FcRn interactions.

To comprehensively characterize Fc-FcRn interactions for research, development and production of therapeutic antibodies and Fc-fusion proteins, a label-free FcRn-dependent transcytosis assay was developed using Madin-Darby Canine Kidney (MDCK) cells stably expressing human FcRn heavy chain and B2M. In this assay, unlabeled test molecules bind to the FcRn expressed on the cell surface at acidic pH, followed by internalization and trafficking across the cell while complexed with FcRn, and finally released into the medium at basic pH. The amount of transcytosed test molecule is quantified by a generic sandwich ELISA.

Here, we demonstrate that an FcRn-mediated transcytosis assay can be used to evaluate FcRn binding of IgG mAbs and Fc-fusion proteins, including wild-type and engineered variants with increased or reduced FcRn binding affinities. Moreover, the assay can assess differential FcRn interactions by oxidized and/or aggregated stressed samples for stability testing. These results show that the FcRn-dependent transcytosis assay is a useful new tool for evaluation of Fc-FcRn interactions and impact of structural changes in Fc-containing molecules on their PK behavior *in vivo*.

2. Materials and methods

2.1. Construction of human FcRn expression vectors and transfection of MDCK cells

MDCK type II cells (Sigma-Aldrich, St. Louis, MO) were maintained in Minimum Essential Media (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT) at 37 °C in 5% CO_2. The medium was changed every other day and the cells passaged once weekly.

Expression vectors encoding the human FcRn heavy chain and a human B2M with C-terminal Myc-Flag tags were purchased from OriGene (Rockville, MD). MDCK II cells were sequentially transfected with expression vectors, first with FcRn then with B2M, using Lipofectamine 2000 (Invitrogen) and selected with 0.5 mg/mL G418 (Invitrogen) for FcRn and 0.25 mg/mL hygromycin B (Invitrogen) for B2M. Clones were analyzed by Western blot for expression of FcRn and B2M proteins and then by FACS for surface expression of FcRn and B2M.

2.2. Test molecules and stressed samples

Test molecules included two humanized IgG1 antibodies (AbX and AbY), one chimeric human IgG1 antibody (AbZ), three humanized IgG1 antibodies with engineered mutations to alter FcRn binding (AbX-TQNA, AbY-HAHQ, AbY-YTE), one Fab (FabX), one recombinant protein without Fc (mA) and three Fc-fusion proteins, all of which were produced in engineered Chinese Hamster Ovary (CHO) cells at Genentech (South San Francisco, CA).

To prepare oxidatively stressed samples, 10% tert-butyl hydroperoxide (tBHP; Sigma Aldrich) was spiked into AbX solutions with final concentrations of 1, 2 or 5% tBHP and then stored at 2–8 °C for 21 h. The oxidized AbX samples were then buffer exchanged to a low ionic strength buffer at pH 6.2 and stored at ≤ −60 °C. Increasing levels of oxidation in treated samples were confirmed with an endoproteinase Lys-C peptide mapping assay using reversed-phase high-performance liquid chromatography. To prepare low-pH stressed samples, AbX was incubated in an acetic buffer pH 3.7 at 40 °C for 7 days. The stressed AbX samples were then buffer exchanged to a regular formulation buffer and stored at ≤ −60 °C. Aggregation levels of the control (mock treated), oxidized, and low-pH stressed samples were assessed by size-exclusion chromatography. The control sample had 7% aggregates and the low-pH stressed sample had 10% aggregates; no increase in aggregation was observed in tBHP-oxidized samples.

2.3. Characterization of transfected MDCK cells

2.3.1. Western blot

MDCK cells expressing human FcRn-FLAG and human B2M-FLAG were lysed in M-PER extraction buffer (Thermo Fisher Scientific; Waltham, MA) supplemented with a protease inhibitor cocktail (Calbiochem; San Diego, CA) for 30 min at 4 °C. The samples were treated with SDS sample buffer and run on a 4–12% Tris Glycine gel (Thermo Fisher Scientific). Non-transfected MDCK cells were used as a negative control. The gel was transferred to a nitrocellulose membrane (Thermo Fisher Scientific), incubated with a mouse anti-FLAG antibody (Sigma Aldrich) and detected with a goat anti-mouse-Europium secondary antibody (Molecular Devices; Sunnyvale, CA). The blot was imaged using a SpectraMax I3 reader system (Molecular Devices).

2.3.2. Flow cytometry

MDCK cells transfected with human FcRn-FLAG and human B2M-FLAG were analyzed *via* flow cytometry. Non-transfected MDCK cells were used as a negative control. FcRn expression was detected with mouse anti-FcRn (Genentech, Inc.) followed by goat anti-mouse Alexa 488 conjugate (Thermo Fisher Scientific). B2M was detected with mouse anti-B2M Alexa 647 conjugate. Cells were fixed in 4% paraformaldehyde. Antibody binding was assessed on a FACS Canto 10 IVD (BD Biosciences; San Jose, CA) and analyzed using FlowJo software (BD Biosciences).

2.3.3. pH-dependent IgG binding

To measure pH-dependent binding of IgG by human FcRn expressed on the cell surface, transfected MDCK cells were incubated for 90 min at 37 °C with10 μg/mL Alexa 647-conjugated AbZ, a chimeric antibody carrying human IgG1-Fc, in Hanks' balanced salt solution (HBSS; Thermo Fisher Scientific) adjusted to either neutral pH (7.4) or acidic pH (5.9). After incubation, the cells were washed twice in pH-adjusted HBSS and fixed in 4% paraformaldehyde and read on a FACS Canto 10 IVD. The fluorescence was measured using FlowJo software. Non-transfected MDCK cells were used as a negative control.

2.4. Transcytosis assay

Cells were seeded at a density of 2×10^6 cells/well in 6-well Transwell plates (Corning Costar, Acton, MA), with 2 and 3 mL of growth medium in the inner and outer chambers, respectively. Cells were used for experiments on the third day post-plating. The integrity of the transfected MDCK cell monolayer in Transwells was evaluated by measuring the trans-epithelial electrical resistance (TEER). The TEER of monolayers before the assay typically ranged from 250 to 300 Ω^*cm^2, a characteristic range for polarized MDCK II cells (Tesar et al., 2006).

Cells were rinsed once with assay buffer consisting of HBSS with 1.3 mM $CaCl_2$ and 0.8 mM $MgSO_4$ (HBSS +, Invitrogen) supplemented with 0.5% teleostean gelatin (Sigma Aldrich) buffered to pH 6.0 (inner chamber) or pH 8.0 (outer chamber). Cells were preincubated with the assay buffer pH 6.0 in inner chamber and pH 8.0 in the outer chamber