Tissue expression profile of human neonatal Fc receptor (FcRn) in Tg32 transgenic mice

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ABSTRACT
The neonatal Fc receptor (FcRn) is a homeostatic receptor responsible for prolonging immunoglobulin G (IgG) half-life by protecting it from lysosomal degradation and recycling it to systemic circulation. Tissue-specific FcRn expression is a critical parameter in physiologically-based pharmacokinetic (PBPK) modeling for translational pharmacokinetics of Fc-containing biotherapeutics. Using online peptide immuno-affinity chromatography coupled with high resolution mass spectrometry, we established a quantitative FcRn tissue protein expression profile in human FcRn (hFcRn) transgenic mice, Tg32 homozygous and hemizygous strains. The concentration of hFcRn across 14 tissues ranged from 3.5 to 111.2 pmole per gram of tissue. Our hFcRn quantification data from Tg32 mice will enable a more refined PBPK model to improve the accuracy of human PK predictions for Fc-containing biotherapeutics.

Abbreviations: PK, Pharmacokinetics; FcRn, neonatal Fc receptor; hFcRn, human FcRn; IgG, immunoglobulin G; IA-LC-HRMS, immuno-affinity liquid chromatography high resolution mass spectrometry; PBPK model, physiologically-based pharmacokinetic model

The neonatal Fc receptor (FcRn) is a heterodimer composed of an α-chain and β2-microglobulin (β2m), known to be involved in various important biological functions throughout human life. One critical function is the FcRn-mediated recycling process, occurring mainly in haematopoietic cells, endothelial and epithelial cells, which maintains serum IgG and albumin homeostasis and influences the circulation half-life of Fc-containing therapeutic proteins. Physiologically-based pharmacokinetic (PBPK) models estimate the PK of drug after its administration. However, a current limitation of using a PBPK model for large molecule PK predictions is the lack of information of localized FcRn concentrations and FcRn recycling rates. Using measured concentrations of therapeutic antibody in plasma and tissues, several recent PBPK reports estimated average mouse FcRn concentrations to range from 20.1 to 49.8 μM. Concurrent with the development of a physiologically-based mechanistic FcRn model, the accuracy of PK projections for monoclonal antibodies and antibody-directed enzyme prodrug therapy have improved. Furthermore, the above model can be extended to include the pharmacodynamic (PD) effect and efficacy following drug administration in order to integrate their prediction with the estimation of PK of therapeutic antibodies. In this study, we experimentally determined tissue-specific hFcRn concentrations in Tg32 transgenic mice, a key missing parameter to be integrated into PBPK models. This data now provides the needed resolution of the PBPK modeling system to the individual tissue level, which is anticipated to improve prediction accuracy and to enable a better a priori understanding of Fc-containing biotherapeutics in vivo.

A quantitative hFcRn tissue assay was developed in Tg32 transgenic mice. The workflow includes tissue extraction, protein precipitation, pellet digestion with trypsin and measurement of a hFcRn tryptic surrogate peptide by peptide immuno-affinity liquid chromatography coupled with high resolution mass spectrometry (IA-LC-HRMS). A polyclonal antibody was generated in rabbits against the hFcRn immunogen peptide, GDDTGVLPTPGEAQADDLK, derived from the cytosolic domain of hFcRn protein. Tg32 mice lack mouse FcRn α-chain and are transgenic for either one (hemizygous) or 2 copies (homozygous) of the hFcRn α-chain under the control of human endogenous promoter. Though sequence similarity between human and mouse β2m is relatively high (77%), the effect of β2m on the PK of a human IgG was investigated in Tg32 mice expressing transgenic hFcRn and mouse endogenous β2m (Tg32) or Tg32 mice expressing both transgenic hFcRn and human β2m in the presence of mouse endogenous β2m (Tg32/hβ2m). The results showed no detectable differences in half-lives between Tg32 and Tg32/hβ2m mice, indicating functional human-mouse hybrid FcRn complexes were formed in the Tg32 transgenic mice systems. PK studies in Tg32 hemizygous mice also showed high correlation of antibody clearance to human (Pearson r = 0.99, p < 0.001), suggesting that Tg32 could serve as an animal model for translational PK of Fc-containing biotherapeutics. Therefore, establishing a quantitative hFcRn protein tissue expression profile in Tg32 will provide a key parameter for translational PBPK models for human PK predictions.
We analyzed a panel of 14 different tissues from hemizygous and homozygous strains of Tg32 transgenic mice (15-18 weeks of age, male, N = 5 each). All procedures were executed within accordance of the Animal Use Protocol (AUP) and adherence to Pfizer institutional animal care and use committees (IACUC) regulations. Tissues were collected 7 weeks after dosing of an IgG at 5 mg/kg. Epididymal fat, spleen, brain, heart, mesenteric lymph node, kidney, liver, lung, pancreas, skeletal muscle of quadriceps, skin, stomach, small intestine, and large intestine were collected, weighted, and snap frozen in liquid nitrogen. The content in stomach, small and large intestine was removed prior to tissue collection. Details of sample preparation, analytical approaches, and assay qualification were previously described. In brief, the assay was qualified with a quantification range of 1.76 – 900 ng/ml, equivalent to 0.89 – 452.70 pmol/g tissue, and a calibration curve was prepared using full length recombinant hFcRn (MW 39.74 kDa) spiked into control mouse liver lysate as an analyte-free, surrogate tissue matrix. A standard curve was obtained by plotting peak area ratio against hFcRn concentration (Fig. S1).

In order to establish extraction reproducibility and understand tissue heterogeneity of hFcRn expression, selected single tissues (lung, muscle, small and large intestine) were dissected into 3-4 pieces for independent lysate preparation. Fig. 1A shows total protein concentration measured by absorbance at 280 nm, and the coefficients of variation (%CVs) were found to be less than 20% from all dissected tissues from both homozygous and hemizygous Tg32 mice. Human FcRn concentrations quantified by IA-LC-HRMS in the lysate from the dissected pieces of each tissue showed little variability in lung (%CV < 20%), whereas muscle showed slightly higher variability (%CV < 30%) (Fig. 1B). Tissues are composed of different cell types, which constitute the tissue architecture and can result in location-dependent heterogeneity of protein expression, leading to variable protein quantification data in dissected tissue samples. FcRn levels in human intestine, measured by ligand binding assay, were reported to be higher in proximal colon compared to jejunum. We also observed differences in hFcRn concentration in dissected pieces of small and large intestine (Fig. 1B), suggesting a higher degree of tissue heterogeneity of hFcRn expression in intestine. In addition to extraction reproducibility, we measured extraction efficiency by digesting with trypsin any tissue debris that remained following the bead homogenization step. Human FcRn peptides recovered from debris were 4.8%, 0.8%, and 0.1%, for lung, liver, and small intestine respectively, compared to hFcRn peptides measured in the extracts from the same tissue. Thus, the hFcRn extraction efficiency was more than 95% from solid tissues into tissue lysate.

With the understanding of quantitative assay performances, including the demonstration of tissue extraction efficiency, reproducibility and bias, a hFcRn tissue expression profile in Tg32 transgenic mice was generated. Human FcRn concentrations obtained from Tg32 homozygous and hemizygous mice are shown in Fig. 2A and Fig. 2B, respectively. The average of hFcRn tissue expression normalized by tissue weight, and the ratio between homozygous and hemizygous are shown in Table 1, where tissues with significant differences in hFcRn expression between both strains are indicated by asterisks. Using Mann-Whitney test to compare the hFcRn concentration obtained in Tg32 hemizygous and homozygous mice, tissues with statistical significant differences of p < 0.05 were lymph node, lung, skin, stomach, heart, kidney, skeletal muscle and brain, indicated by double asterisks (**), whereas tissues with statistical significant differences of p < 0.1 were liver and large intestine, indicated by single asterisks (*). No significant difference was observed in spleen, small intestine and epididymal fat. The average hFcRn expression ratio between homozygous and hemizygous Tg32 mice strains was 1.95 fold. Human FcRn expression in Tg32 mice pancreas was below the limit of quantification, but detectable from tissue matrices (Fig. S2), suggesting hFcRn concentration in pancreas was less than 0.89 pmol/g of tissue. Human FcRn tissue expression normalized by total protein concentration is shown in Table S1, and tissue protein concentration determined by absorbance at 280 nm is shown in Table S2. Though there are 2 copies of hFcRn α-chain genes in the homozygous mouse strain, the expression quantity of endogenous mouse β2m (mβ2m) may become a limiting factor in the formation of an active FcRn complex. The functionality of IgG binding to hybrid FcRn complex in transgenic mouse carrying multiple copies of bovine FcRn or human FcRn suggested active FcRn complex formation with mβ2m. Quantitative evidence of β2m expression level came from the study of human trophoblast-derived cells, which total protein expression of endogenous β2m increased by 120% comparing hFcRn overexpression cells and the parental cells using immunoblotting analysis. It will be of interest to pursue and understand the expression level of tissue-specific mβ2m as well as other factors that result in the hFcRn expression differences in the 2 genotypes of the Tg32 strains.

In the measurement of hFcRn expression across 14 tissues, lymph node, lung and skin showed highest expression of hFcRn (range of 33.3 – 111.2 pmol/g tissue). The high expression of hFcRn in lung has been considered a key factor for investigating a pulmonary delivery route for Fc-fusion proteins or monoclonal antibodies. Delivery of erythropoietin-Fc (EpoFc) fusion protein by inhalation in a human Phase 1 clinical study showed ~70% of the Epo-Fc was delivered safely and effectively to the central lung region. Furthermore, a mouse FcRn-incorporated PBPK model predicted that the skin could account for 33% of the total IgG elimination. Our data support this prediction by taking into account that skin takes a large percentage of body weight. Unlike other tissues, FcRn expression in the brain has been in dispute. Studies from blood and brain tissue analysis of 125I-labeled IgG intravenously administered into WT and FcRn KO mice showed no significant differences in the AUC (area under the curve of IgG concentration) ratio between brain and blood. Interestingly, studies using methods to localize dosed antibodies via immunohistochemistry and confocal microscopy of antibody against rat FcRn α-chain in rat brain seemed to suggest that FcRn enabled the transport of monoclonal antibodies across the blood-brain barrier. The likely mechanism is reverse transcytosis in brain microvascular endothelial cells that form the blood brain barrier, enabling the efflux of IgG from the brain. We used our IA-LC-HRMS approach to measure hFcRn expression in the Tg32 mice brain. Although hFcRn concentrations were relatively low compared to other tissues, they were clearly quantifiable in hemizygous (3.5 pmol/g of tissue) and homozygous (8.0 pmol/g of tissue) mice. In future...
studies, it will be of interest to establish whether FcRn expression in the human brain is comparable to the transgenic mice. In a systemic administration study, the contribution of hFcRn on IgG tissue distribution has been reported by co-dosing $^{125}$I-labeled IgG and $^{111}$In-labeled IgG indicating that liver, spleen, skin and muscle play important roles in IgG catabolism. Our

Figure 1. Tissue extraction reproducibility and heterogeneity from independent lysate preparation of dissected tissue pieces resulting from the same lung, muscle, small and large intestine samples from both homozygous and hemizygous mice showing (A) protein concentration and (B) hFcRn expression. Protein concentration was determined by absorbance at 280 nm.
report and previous studies show important connections between FcRn expression and the understanding of biodistribution for therapeutic monoclonal antibodies, antibody-drug conjugates, Fc-fusion and albumin fusion proteins.

FcRn measurements in some selected tissues have previously been carried out via ligand binding assay, protein immunoblotting, tissue staining, or transcriptional analysis from various cultured cell types. However, to the best of our knowledge, systematic quantification of FcRn across tissues has not been reported, which is likely due to the technical challenges associated with tissue-based bioanalysis of membrane proteins.

The challenges for endogenous membrane protein quantification across various tissue types include maximizing tissue extraction efficiency, ensuring high assay sensitivity, avoiding tissue matrices interference, and establishing rigorous quantification performances of the assay. Our quantification strategy by protein pellet digestion and online peptide immunofinity enrichment is suitable for endosomal and cell-surface hFcRn, i.e., total hFcRn quantification, bypassing challenges of protein enrichment in a membranous environment using anti-FcRn antibodies. Such an approach coupled with high resolution mass spectrometry has enabled this tissue-based hFcRn

Figure 2. Human FcRn tissue expression profile in (A) Tg32- homozygous and (B) Tg32-hemizygous mice.
Table 1. FcRn tissue expression of human FcRn transgenic mice, Tg32, normalized by tissue weight. (N = 5) Using Mann-Whitney test, tissues with p < 0.05 are indicated by double asterisks (**); p < 0.1 are indicated by single asterisks (*).

| Tissue type         | Homozygous | Hemizygous | FcRn expression ratio (Homo/Hemi) | P value of Mann-Whitney test |
|---------------------|------------|------------|----------------------------------|-----------------------------|
| Lymph node**        | 111.2      | 33.25      | 3.34                             | 0.008                       |
| Lung**              | 98.30      | 50.04      | 1.96                             | 0.016                       |
| Skin**              | 95.56      | 40.92      | 2.34                             | 0.008                       |
| Liver*              | 77.64      | 55.38      | 1.40                             | 0.095                       |
| Spleen              | 72.40      | 49.97      | 1.45                             | >0.999                      |
| Large Intestine*    | 59.45      | 23.23      | 2.56                             | 0.057                       |
| Small intestine     | 56.77      | 50.34      | 1.13                             | 0.629                       |
| Stomach*            | 37.56      | 18.01      | 2.09                             | 0.032                       |
| Epididymal fat      | 30.02      | 26.18      | 1.15                             | 0.222                       |
| Heart**             | 27.90      | 14.91      | 1.87                             | 0.008                       |
| Kidney**            | 24.74      | 13.64      | 1.81                             | 0.032                       |
| Skeletal muscle**   | 15.67      | 8.010      | 1.96                             | 0.008                       |
| Brain**             | 7.995      | 3.509      | 2.27                             | 0.008                       |

Note: Data for human FcRn expression in Tg32 hemizygous mouse tissues of skeletal muscle, heart, stomach, large and small intestine, and liver previously reported in ref. 13.

Materials and methods

Material, methods and associated references are available as supplementary material.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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