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Research article

# The role of Fc-receptors in the uptake and transport of therapeutic antibodies in the retinal pigment epithelium

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ABSTRACT

In the ophthalmological clinic, intravitreally applied antibodies or Fc-containing fusion proteins are frequently used, but the biology and pharmacokinetics of these therapeutics in the retina are not well understood. We have previously shown intracellular uptake of Fc-containing molecules in RPE cells. In this study, we investigated the involvement of Fc-receptors, both Fcγ-receptors and the neonatal Fc-receptor (FcRn) in the uptake and intracellular trafficking of the VEGF-antagonists bevacizumab, aflibercept and the anti-CD20 antibody rituximab in three different model systems, primary porcine RPE cells, ARPE-19 cells and porcine RPE/choroid explants.

The expression of Fcγ-receptors was tested in primary porcine RPE cells, and the expression of Fcγ-receptors I and II could be shown in RT-PCR and qRT-PCR, while the expression of FcRn was additionally confirmed in Western blot and immunocytochemistry. All three compounds, bevacizumab, rituximab and aflibercept, were taken up into the cells and displayed a characteristic time-dependent pattern, as shown in Western blot and immunohistochemistry. The uptake was not altered by the inhibition of Fcγ-receptors using different inhibitors (TruStain FcX, genistein, R406). However, the inhibition of FcRn with an antagonistic antibody reduced intracellular IgG in porcine RPE cells (rituximab) and ARPE-19 cells (bevacizumab, rituximab). Colocalisations between the tested compounds and myosin7a could be found. In addition, limited colocalization with FcRn and the tested compounds, as well as triple localization between compound, FcRn and myosin7a could be detected, indicating a role of myosin7a in FcRn mediated transport. However, the colocalizations are restricted to small fractions of the Fc-containing compounds. Furthermore, the FcRn is mainly found in the membrane section, where only minute amounts of the Fc-containing compounds are seen, suggesting a limited interaction. An apical to choroidal transport of IgG through the RPE/choroid can be found in RPE/choroid explants. Inhibition of FcRn increases the amount of bevacizumab found on the choroidal side, suggesting a role of FcRn in the recycling of bevacizumab.

In conclusion, our data indicate a role for FcRn, but not Fcγ-receptors, in the uptake and transport of Fc-containing molecules in the RPE and indicate a recycling function of FcRn in the retina.

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## 1. Introduction

In the ophthalmological clinic, intravitreally applied antibodies or Fc-containing fusion proteins are frequently used. Major therapeutic application is the intravitreal injection of bevacizumab or aflibercept for the treatment of age-related macular degeneration or diabetic macular edema (Volz and Pauly, 2015), but also other therapeutic antibodies, e.g. infliximab for uveitis (Takeuchi, 2013) or rituximab for the treatment of vitreoretinal lymphoma (Larkin et al., 2014). The biology and pharmacokinetics of these

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therapeutics in the retina are not well understood.

RPE cells are situated in the interface between the retina and the choroid and exert important functions in the maintenance (Strauss, 2005) and the immunological regulation of the retina (Klettner, 2015). We have previously shown that bevacizumab and aflibercept are taken up by retinal pigment epithelial (RPE) cells (Klettner et al., 2009, 2014bib_Klettner_et_al_2014), and that bevacizumab is likely to be transported via myosin7a along actin filaments, and secreted in exosomes in these cells (Aboul Naga et al., 2015). The data indicate that RPE cells may be important protagonists in the clearance of therapeutic antibodies from the subretinal space into the choroid.

Studies have indicated that the Fc-portion of these therapeutic molecules is of importance for their intracellular presence and transportation, as indicated by similar findings for RPE uptake of bevacizumab and aflibercept (Klettner et al., 2014; Julien et al., 2014) as well as systemic availability of these molecules (Klettner, 2014).

The main receptors for antibodies (and Fc-fragment containing fusion proteins) are the Fc-receptors, which in the case of IgG consist of the Fcγ-receptors (FcγR), expressed on the cell membrane, and the intracellularly expressed neonatal Fc-receptor (FcRn). FcγR can be divided in FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). FcγRI is considered a high affinity receptor which binds monomeric IgG, while FcγRII and FcγRIII are considered low affinity receptors which recognize mainly immune receptor complexes (Nimmerjahn and Ravetch, 2008). All FcγR exert their function via tyrosine phosphorylation, either via immunoreceptor tyrosine based activation motif (ITAM) or immunoreceptor tyrosine based inhibition motif (ITIM) intracytoplasmic domain. In particular, the tyrosine activation is needed for FcγR mediated internalization (Greenberg et al., 1993; Devriendt et al., 2013; Huang et al., 2006). The expression of Fcγ receptors has been shown for the immortal cell line ARPE-19 (Wang et al., 2010), which is a generally used RPE model (Dunn et al., 1996), but little is known about their expression in primary RPE (Elner et al., 1981; Eckhert and Hafeman, 1986; Dutt et al., 1989).

In contrast to the FcγR, FcRn is an intracellular receptor which binds to IgG in intracellular vesicles at low pH (<6.5). While originally described to transfer IgG from the mother to the infant via the placenta (Raghavan et al., 1994), FcRn is vital for the extension of the serum half-life of antibodies in the circulation and protecting it from lysosome degradation (Simister et al., 1997). Hence, FcRn is an important factor in systemic antibody pharmacokinetics (Vaughn et al., 1997). The presence on FcRn on RPE cells has recently been shown (Powner et al., 2014; van Bilsen et al., 2011).

In this study, we have investigated the expression of Fc-receptors in the porcine RPE, the involvement of Fc-receptors in the uptake of Fc-containing therapeutic molecules, and the involvement of Fc-receptors in their transportation. Our data indicate a role of FcRn but not FcγR in antibody pharmacokinetics.

## 2. Material and methods

### 2.1. Primary porcine RPE cells

Primary porcine RPE were isolated as described elsewhere (Klettner and Roider, 2008). In brief, eyes of freshly slaughtered pigs were cleaned of adjacent tissue, briefly incubated in antiseptic solution and the anterior part of the eye was removed. In each eye cup, trypsin (PAA, Cölbe, Germany) was added, and incubated for 10 min at 37°. Trypsin solution was removed and substituted with trypsin-EDTA (PAA) for 45 min at 37°. RPE cells were gently pipetted of the choroid, collected in media and washed. RPE of 6–7 eyes were collected and seeded in a 60 mm dish (Thermo Scientific, Dreirich, Germany). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, PAA) and Ham F12 medium (PAA) (1:1) supplemented with penicillin/streptomycin (1%), L-glutamine, amphotericine B (0.5 μg/ml), HEPES (25 mM), sodium-pyruvate (110 mg/ml) (all PAA) and 10% fetal calf serum (Linaris, Wertheim-Bettingen, Germany). For microscopy, cells were cultivated to confluence on collagen-coated (Collagen A, Biochrome, Berlin, Germany) cover slips (21 × 26 mm, Menzel GmbH, Braunschweig, Germany).

### 2.2. ARPE-19

ARPE-19 cells (ATCC, Wesel, Germany), an immortal human RPE cell line, were cultivated in Dulbecco's modified Eagle's medium (DMEM; PAA), supplemented with penicillin/streptomycin (1%), non-essential amino acids (1%) (both PAA), and 10% fetal calf serum (Linaris).

### 2.3. RPE/choroid organ culture

Porcine RPE/choroid organ cultures were prepared as previously described with modifications (Treumer et al., 2012). In brief, preparation from eyes of freshly slaughtered pigs was initiated within 4 h of death. The globes were briefly immersed in antiseptic solution (betaisodona, Mundipharma, Limburg, Germany). Anterior segment, vitreous and retina were removed and the RPE/choroid layer was gently separated from the sclera using forceps and scissors. The RPE-choroid explant was fixed in a fixation ring and rings were transferred to culture medium in multiwell culture plates (Corning Costar, Lowell, USA). The culture medium was a mixture of equal amounts of Dulbecco's modified Eagle's medium (DMEM; PAA) and Ham-F12 medium (PAA), supplemented with penicillin/streptomycin (1%) (PAA), l-glutamine (PAA), 10% fetal calf serum (Linaris), taurine (100 μM), and calcium (2 mM) (both Sigma–Aldrich, Steinheim, Germany). The cultures were brought into a modified Ussing chamber with an exposed tissue diameter of 7 mm. This chamber allows the separation of apical (RPE-facing) and basal (choroid facing) compartments. To evaluate viability of the cultures, a calcein (MoBiTec, Göttingen, Germany) stain was conducted as previously described (Klettner et al., 2013). Only viable cultures were further analyzed.

### 2.4. Peripheral mononuclear blood cells

As a positive control for the expression of Fcγ receptors, porcine peripheral mononuclear blood cells (PBMC) were used. Fresh porcine blood was obtained from a local slaughterhouse and PBMC were collected as previously described (Berg et al., 2013).

### 2.5. RNA preparation

Total RNA for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was prepared using a Nucleospin RNA Kit (Machery-Nagel, Düren, Germany) according to the manufacturers' instruction. Total RNA for quantitative real-time-PCR (qRT-PCR) was isolated by TRIZOL Reagent (Life Technologies, Darmstadt, Germany) as previously described (Klettner et al., 2001).

### 2.6. RT-PCR

The expression of FcγRI, II and III, and FcRn was evaluated by PCR. Beta-actin expression was investigated as control. The specificity of the primers was confirmed by sequencing of the PCR product (SeqLab, Göttingen, Germany) and sequence alignment (EMBOSS Pairwise Alignment Algorithms). All primer sequences

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