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Research article

Neonatal Fc receptor FcRn is involved in intracellular transport of the Fc fusion protein aflibercept and its transition through retinal endothelial cells

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ABSTRACT

Retinal endothelial cells (REC) likely contribute to the clearance of intravitreally injected IgG. Because this is of high relevance to the pharmacokinetic assessment of the widely used therapeutic Fc fusion protein aflibercept, we studied its transport through immortalized bovine REC (iBREC) in detail. For shuttling of IgG or Fc fusion proteins like aflibercept, endothelial cells use the highly conserved neonatal Fc receptor (FcRn) also expressed in iBREC where it is down regulated by serum depletion. Therefore, we focused on studying intracellular localization and transport of aflibercept under conditions affecting its interaction with the FcRn. Intracellular localization of aflibercept was assessed by Western-blot analyses of subcellular protein fractions or by immunofluorescence staining. After uptake in a temperature-dependent process, aflibercept co-localized with early endosomes, which harbor FcRn. Similar amounts of aflibercept were co-extracted with proteins from membranes/organelles irrespectively of the amount of FBS in the culture medium. Lowering the concentration of FBS resulted in a strong, but reversible association with cytoskeletal proteins suggesting a block in intracellular transport. In accordance with this finding, aflibercept's transport through an iBREC monolayer grown on porous membrane inserts was markedly delayed in the absence of FBS in the culture medium indicating that aflibercept is taken up but not exocytosed under these conditions. Transcytosis of aflibercept was also strongly delayed by inhibition of phosphatidylinositol 3-kinase with LY294002, which affects FcRn-mediated IgG transport. A similar inhibition of aflibercept's transport was observed with IgG-binding proteins (i.e. protein A or protein G) that block interaction between FcRn and aflibercept. Interfering with aflibercept's binding to the FcRn with protein A (or protein G) or the inhibitory FcRn-specific monoclonal antibody 1G3 resulted in a reduced amount of intracellular aflibercept. Taken together, our results strongly suggest that FcRn is involved in transport of aflibercept through REC in vitro.

1. Introduction

Pathogeneses of diabetic retinopathy, retinal vein inclusion and age related macular edema are associated with de-regulated expression of various cytokines including the most relevant vascular endothelial growth factor A (VEGF-A). Accordingly, VEGF-binding proteins ranibizumab, aflibercept and bevacizumab are now widely used to treat these conditions, and their VEGF-inactivating activities were clearly confirmed by in vitro studies with retinal endothelial cells (REC) (Presta et al., 1997; Holash et al., 2002; Ferrara et al., 2006, Arevalo et al., 2011, Do et al., 2012; Lang et al., 2013; Deissler et al., 2008, 2011, 2012, 2014; Stewart et al., 2011). The pharmacokinetic profiles of therapeutic IgG, their derivatives and fragment crystalisable (Fc) fusion proteins are determined by their clearance from the eye, a process in which REC seem to play a role (Kim et al., 2009; Julien et al., 2014). We have recently shown that immortalized endothelial cells of the bovine retina (iBREC) can take up the humanized VEGF-binding antibody bevacizumab and transport it through the monolayer or release it from

Abbreviations: DMSO, dimethyl sulfoxide; EEA1, early endosome antigen 1; Fc, fragment crystalisable; FcRn, neonatal Fc receptor; FBS, fetal bovine serum; (i)BREC, (immortalized) bovine retinal endothelial cells; PI3-kinase, phosphatidylinositol 3-kinase; MAb, monoclonal antibody; PBSd, phosphate buffered saline without Ca^{2+} and without Mg^{2+} ions; REC, retinal endothelial cells; RPE, retinal pigment epithelium; TJ, tight junction; VEGF(-A), vascular endothelial growth factor (A).

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the uptake side (Deissler et al., 2016). Blocking the interaction of the IgG with the neonatal Fc receptor (FcRn) impaired transport of bevacizumab and affected its subcellular localization in these cells (Deissler et al., 2016). FcRn, which is expressed in various ocular structures including the retinal endothelium, is primarily involved in transcytosis and recycling of IgG and albumin in endothelial cells of various origins and responsible for maintaining homeostasis of these proteins in the serum (Kim et al., 2008; Powner et al., 2014; Junghans and Anderson, 1996; Ward et al., 2003). This receptor is a dimer consisting of two not covalently linked subunits: β_2-microglobulin and a larger polypeptide related to major histocompatibility complex class I proteins (Junghans and Anderson, 1996). In early endosomes, which are characterized by the expression of the early endosome antigen 1 (EEA1), the FcRn binds IgG and albumin independently from each other at pH 6. The complexes are then shuttled away from the lysosomes and both proteins are released by exocytosis at physiological pH (Junghans and Anderson, 1996; Ward et al., 2003, 2005; Ober et al., 2004; Goebl et al., 2008; Jerdeva et al., 2010, Sand et al., 2015). Interfering with vesicle formation by blocking phosphatidylinositol 3-kinase (PI3-kinase) with LY294002 or Wortmannin strongly abolished FcRn-mediated IgG transport in rat kidney epithelial cells expressing FcRn (McCarthy et al., 2000). The human and bovine homologues of FcRn are highly conserved, and it was confirmed in several studies that human IgG is indeed efficiently bound, transported and released in the transcytosis pathway by bovine FcRn (Ober et al., 2001; Kacskovics et al., 2000, 2006; Cui et al., 2014). Expression of the FcRn in iBREC is exclusively associated with proteins from the membrane/organelle fraction, is weaker in the absence of FBS but not affected by bevacizumab. Most interestingly, transport of bevacizumab through an iBREC monolayer is retarded in the absence of FBS accompanied by its accumulation in the fraction of cytoskeleton proteins (Deissler et al., 2016). iBREC also take up aflibercept, which consists of binding domains of VEGF receptors and the human IgG1 Fc domain, within a few hours after exposure, and the Fc fusion protein is then mainly localized in a perinuclear region (Holash et al., 2002; Deissler et al., 2014). These observations invited the hypothesis that binding to FcRn might also be involved in aflibercept's intra- and transcellular transport and we therefore studied uptake and transport of aflibercept under conditions affecting its potential interaction with FcRn.

2. Materials and methods

2.1. Aflibercept and antibodies

Eylea (40 mg/ml aflibercept) was a kind gift from Bayer Vital GmbH (Leverkusen, Germany) (Holash et al., 2002; Do et al., 2012). Goat polyclonal detection antibodies ($F(ab')_2$, labelled with Alexa Fluor 594/488) were from Thermo Scientific (Karlsruhe, Germany); other antibodies are listed in Table 1.

2.2. General information on cell culture, treatment of iBREC with effectors and transcytosis assay

Only general information is provided here, because the current study is based on an approach already described in great detail (Deissler et al., 2016). Confluent monolayers of telomerase-immortalized bovine microvascular REC (iBREC) established after cultivation for at least 4 d were used in all experiments, which were repeated at least twice (Deissler et al., 2005, 2008, 2011, 2016). In control experiments, cells were processed identically in medium only lacking the effector(s) under investigation. A final concentration of 250 μg/ml (~2 μM) aflibercept - achievable by intravitreal injection - was always used.

To study the effect of FBS on internalization or transport of aflibercept, iBREC were incubated with culture medium containing 0%, 1% or 5% FBS for 24 h before they were exposed to aflibercept for up to 1 d (Deissler et al., 2014, 2016). Potential competitive effects of Fc-binding proteins (i.e. protein A and protein G; Thermo Scientific) on the internalization or transport of aflibercept were explored as described (Raghavan et al., 1994; Deissler et al., 2016). To prevent interaction of aflibercept with FcRn, iBREC were pre-treated with 10 μg/ml of mouse monoclonal antibody (MAb) 1G3 for 1 h before aflibercept was added for additional 4 h. Whole cell extracts and subcellular fractions, i.e. proteins localized in the cytoplasm, in membranes/organelles, and components of the cytoskeleton, were prepared from fresh or frozen cell pellets as described in detail elsewhere (Deissler et al., 2012, 2014, 2016).

Transcytosis assays to assess transport of aflibercept from the lower to the upper chamber through a confluent iBREC monolayer cultivated on membrane inserts (4.7 cm^2, pore size 0.4 μm; Corning) were performed as published (Antohe et al., 2001; Deissler et al., 2016). The effect of inhibition of PI3-kinase on aflibercept transport was evaluated by adding LY294002 (500 nM in 0.01% dimethyl sulfoxide (DMSO); Sigma-Aldrich, Dreieich, Germany) to both chambers. After pre-treating iBREC for 2 h, aflibercept was placed in the bottom chamber and samples were then taken from the upper chamber.

All Western blot analyses were performed under reducing conditions; equal amounts of protein were loaded per lane when cell extracts (~25 μg) or fractions thereof (~5 μg) were analyzed (Deissler et al., 2012, 2014). To determine the amount of transported aflibercept, the sample (75 μl) taken from the supernatant in upper chamber (total volume: 1.5 ml) was directly analyzed by Western blot without further processing; 0.1% of the supernatant was loaded per lane (Deissler et al., 2016). Western blot analyses were repeated at least twice; a typical image is shown. Observed differences

Table 1
Antibodies used in study.

Target	Specificity	Host	Source	Concentration
FcRn (large chain)	human, bovine	rabbit, polyclonal	bio-techne (Wiesbaden, Germany), NBP1-89127	Western blot analysis: 3 μg/ml immunofluorescence staining: unspecific
FcRn (heterodimer)	human	mouse, monoclonal	Genetex (Biozol, Eching, Germany), 1G3	functional assay: 10 μg/ml
EEA1	human, bovine	rabbit, polyclonal	abcam (Cambridge, UK), ab137403	Western blot analysis: 70 ng/ml, immunofluorescence staining: 20 μg/ml
claudin-1	human, bovine	rabbit, polyclonal	Thermo Scientific, JAY.8	Western blot analysis: 100 ng/ml JAY.8 does not cross react with claudin-3
actin	human, bovine	mouse, monoclonal	abcam, AC-40	Western blot analysis: 500 ng/ml
IgG, γ-chain	human	goat, polyclonal, coupled to HRP[a]	Thermo Scientific, 628420	Western blot analysis: 1:20000
whole IgG	rabbit	goat, polyclonal, coupled to HRP[a]	Biorad (Munich, Germany), 170-5046	Western blot analysis: 1:30000
whole IgG	mouse	goat, polyclonal, coupled to HRP[a]	Biorad, 170-5047	Western blot analysis: 1:20000

[a] HRP: horseradish peroxidase.