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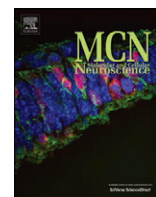

Transferrin receptor expression and role in transendothelial transport of transferrin in cultured brain endothelial monolayers

Maria Hersom [a], Hans Christian Helms [a], Natasia Pretzer [a], Charlotte Goldeman [a], Andreas I. Jensen [b], Gregory Severin [b], Morten S. Nielsen [c], René Holm [a,d], Birger Brodin [a,*]

[a] Department of Pharmacy, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark
[b] Center for Nuclear Technologies, Technical University of Denmark, Frederiksborgvej 339, 4000 Roskilde, Denmark
[c] Department of Biomedicine, Aarhus University, Ole Worms Allé 3, 8000 Aarhus C, Denmark
[d] Pharmaceutical Science and CMC Biologics, H. Lundbeck A/S, 2500 Valby, Denmark

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ABSTRACT

Receptor-mediated transcytosis of the transferrin receptor has been suggested as a potential transport system to deliver therapeutic molecules into the brain. Recent studies have however shown that therapeutic antibodies, which have been reported to cross the brain endothelium, reach greater brain exposure when the affinity of the antibodies to the transferrin receptor is lowered. The lower affinity of the antibodies to the transferrin receptor facilitates the dissociation from the receptor within the endosomal compartments, which may indicate that the receptor itself does not necessarily move across the endothelial cells by transcytosis. The aim of the present study was to investigate transferrin receptor expression and role in transendothelial transferrin transport in cultured bovine brain endothelial cell monolayers.

Transferrin receptor mRNA and protein levels were investigated in endothelial mono-cultures and co-cultures with astrocytes, as well as in freshly isolated brain capillaries using qPCR, immunocytochemistry and Western blotting. Transendothelial transport and luminal association of holo-transferrin was investigated using [^{125}I]holo-transferrin or [^{59}Fe]-transferrin.

Transferrin receptor mRNA expression in all cell culture configurations was lower than in freshly isolated capillaries, but the expression slightly increased during six days of culture. The mRNA expression levels were similar in mono-cultures and co-cultures. Immunostaining demonstrated comparable transferrin receptor localization patterns in mono-cultures and co-cultures. The endothelial cells demonstrated an up-regulation of transferrin receptor mRNA after treatment with the iron chelator deferoxamine. The association of [^{125}I]holo-transferrin and [^{59}Fe]-transferrin to the endothelial cells was inhibited by an excess of unlabeled holo-transferrin, indicating receptor mediated association. However, over time the cell associated [^{59}Fe]-label exceeded that of [^{125}I]holo-transferrin, which could indicate release of iron in the endothelial cells and receptor recycling. Luminal-to-abluminal transport of [^{125}I]holo-transferrin across endothelial cell monolayers was low and not inhibited by unlabeled holo-transferrin. This indicated that transendothelial transferrin transport was independent of transferrin receptor-mediated transcytosis.

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1. Introduction

The blood-brain barrier (BBB) limits the transport of macromolecules from the systemic circulation into the brain, thereby posing a major challenge for brain delivery of larger (>500 Da) therapeutic molecules (Lajoie and Shusta, 2015). Several endogenous macromolecules with proposed physiological roles in the central nervous system have been suggested to cross the brain endothelium *via* binding to receptors on the luminal membrane, followed by vesicular trafficking and subsequent release at the abluminal membrane. This process is commonly referred to as receptor mediated transcytosis (Predescu et al., 2007; Tuma and Hubbard, 2003). The transferrin receptor (TfR), a transmembrane glycoprotein consisting of two 90 kDa subunits, has actively been explored as a potential transport system to deliver therapeutic molecules into the brain (for references see Lajoie and Shusta, 2015; Yu and Watts, 2013). Recent studies have reported brain delivery of TfR bispecific antibodies or antibody constructs with targeting Fab fragments in nonhuman primates and Alzheimer's mice models respectively (Niewoehner et al., 2014; Yu et al., 2014).

The physiological role of the TfR in the brain endothelium is to mediate the cellular uptake of circulating iron bound to transferrin (Tf)

* Corresponding author at: Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark.
E-mail address: birger.brodin@sund.ku.dk (B. Brodin).

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and mediate delivery of iron to the brain (Fishman et al., 1987; Jefferies et al., 1984; Pardridge et al., 1987). The precise cellular mechanism for the TfR trafficking and how iron enters the brain *via* the BBB is, however, still not mapped. It has been proposed that iron transport occurs *via* receptor mediated transcytosis of holo-Tf (Descamps et al., 1996; Fishman et al., 1987). Iron has however also been shown to dissociate from Tf in the acidified endosomes and a number of recent reports have shown that Tf itself does not cross the intact BBB (for references see Lichota et al., 2010; McCarthy and Kosman, 2015). Furthermore, recent studies have shown that the therapeutic antibodies which have been reported to cross the brain endothelium reaches greater brain exposure when the affinity of the antibodies to the TfR is lowered, thereby facilitating the dissociation from the TfR within the endosomal compartments (Bien-Ly et al., 2014; Yu et al., 2011). This may indicate that the receptor itself does not necessarily move across the endothelial cells by transcytosis. The limited understanding of the cellular route by the TfR thus makes it a challenge to design therapeutics utilizing the receptor system as a transport pathway.

Brain capillary endothelial cells cultured on permeable supports form monolayers that can be used to investigate transendothelial transport processes. A wide range of species including mouse, rat, pig and human have been used to generate these *in vitro* BBB models (for references see Helms et al., 2016). The cultured monolayers should possess high junctional tightness in order to minimize paracellular transport of test compounds. Primary endothelial cells of bovine and porcine origin are among the most commonly used in the generation of electrically tight monolayers, either alone or in co-culture with astrocytes (for references see Helms et al., 2016). Studies from our group have demonstrated that bovine endothelial cells, cultured in media with a high buffer capacity develop high transendothelial electrical resistance (TEER) resulting in low permeability of small hydrophilic compounds (Helms et al., 2010). Furthermore, this cell culture model has been shown to express BBB marker proteins such as tight junction proteins, as well as vectorial transport of efflux transporter substrates (Helms et al., 2014, 2010). The TfR expression and transendothelial transport has previously been investigated in bovine BBB *in vitro* models (Descamps et al., 1996; Raub and Newton, 1991; Visser et al., 2004a,b). These studies demonstrated TfR expression and TfR transcytosis to different degrees. However, as these model configurations did not demonstrate high electrical tightness, potential paracellular transport could have occurred. The aim of the present study was to evaluate the TfR expression and transport capacity in an electrically tight model with corresponding low paracellular permeability and to address whether Tf is likely to undergo receptor transcytosis across brain endothelial cells.

Using qPCR and confocal microscopy it was shown that the TfR was expressed and that the presence of astrocytes had no effect on the TfR expression. Using [^{125}I]holo-Tf and [^{59}Fe]-Tf it was demonstrated that TfR was functionally active and able to mediate uptake of Tf. Uptake of [^{59}Fe]-Tf exceeded that of [^{125}I]holo-Tf. Transport across the endothelial monolayers was low and could not be inhibited by an excess of unlabeled Tf.

The findings indicated that the TfR facilitated luminal uptake of Tf but not transcellular transport in cultured bovine brain endothelial cells.

2. Materials and methods

2.1. Materials

Culture media compositions are listed in Table 1. Fetal Bovine Serum (FBS) was from PAA-Laboratories (Pasching, Austria), Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), puromycin dihydrochloride, dextran (31392), heparin, bovine holo-transferrin and all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Table 1
Culture media compositions.

Abbreviation	Media	Content
DMEM	Complete Dulbecco's Modified Eagle's Medium	DMEM-AQ Fetal Bovine Serum (FBS) 10% Non-essential amino acids (×100) 10 mL/L Penicillin/streptomycin solution 100 U/mL/100 μg/mL
GM−	Growth medium −	DMEM Heparin 125 μg/mL
GM+	Growth medium +	DMEM:ACM (1:1) Heparin 125 μg/mL
ACM	Astrocyte-conditioned medium	DMEM removed from cultured astrocytes after 2 days of culture
DM	Differentiation medium	Powdered DMEM 13.3 g/L TES 11.5 g/L (50 mM) Non-essential amino acids (×100) 10 mL/L Penicillin/streptomycin solution 100 U/mL/100 μg/mL L-Glutamine 2 mM FBS 10% 8-(4-CPT)cAMP 312.5 μM Dexamethasone 0.5 μM RO-20-1724 17.5 μM

FBS = Fetal Bovine Serum, cAMP = cyclic adenosine monophosphate, TES = *N*-Tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid.

2.2. Antibodies and primers

All primers in Table 2 were purchased from Thermo Fisher Scientific. α-von Willebrand Factor (ab6994) was from Abcam (Cambridge, UK) and α-β-actin (A5441) was from Sigma-Aldrich. α-transferrin receptor (13-6800), propidium iodide, Alexa 488 conjugated phalloidin (A12379), Alexa 488 conjugated goat α-rabbit IgG (A11008), Alexa 488 conjugated goat α-mouse IgG (A11001), HRP conjugate goat α-mouse (62-6520) and goat α-rabbit (G-21234) were from Thermo Fisher Scientific.

2.3. Isolation of bovine capillaries

Brain capillaries of bovine origin were isolated as described previously (Helms and Brodin, 2014). For preparations of capillaries for immunocytochemistry and quantitative polymerase chain reaction (qPCR), the protocol was stopped just before the addition of digestion enzymes and the capillaries were spun down in 16% dextran (M_r 450,000–650,000) in DMEM for 15 min at 2630 ×*g* followed by a 10 min spin at 485 ×*g* in DMEM.

Table 2
Primers used in the study. The primers were selected to match the bovine homologues using SDSC Biology Workbench 3.2. As negative controls, the primer sets were tested on RNA isolated from rat astrocytes in a manner similar to the procedure described for endothelial cells, which verified that they did not produce products in the relevant cycle range (data not shown).

Overview of applied primers			
Primers			
Target	Forward strand	Reverse strand	Product size
TfR	TTTAGTCTGGCTCGGCAAGT	CGGTTTTGCGATACTGGTTT	120
HPRT1	CGTGGTGATTAGCGATGATG	TTCATCACATCTCGAGCCAG	144
β-Actin	AGGCTGTGCTGTCCCTGTAT	AGGTAGTTTCGTGAATGCCG	426

TfR transferrin receptor, HPRT1 hypoxanthine phosphoribosyltransferase 1, β-actin beta-actin.

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