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The blood–brain barrier transmigrating single domain antibody: mechanisms of transport and antigenic epitopes in human brain endothelial cells

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Abstract

Antibodies against receptors that undergo transcytosis across the blood–brain barrier (BBB) have been used as vectors to target drugs or therapeutic peptides into the brain. We have recently discovered a novel single domain antibody, FC5, which transmigrates across human cerebral endothelial cells *in vitro* and the BBB *in vivo*. The purpose of this study was to characterize mechanisms of FC5 endocytosis and transcytosis across the BBB and its putative receptor on human brain endothelial cells. The transport of FC5 across human brain endothelial cells was polarized, charge independent and temperature dependent, suggesting a receptor-mediated process. FC5 taken up by human brain endothelial cells co-localized with clathrin but not with caveolin-1 by immunochemistry and was detected in clathrin-enriched subcellular fractions by western blot. The transendothelial migration of FC5 was reduced by inhibitors of clathrin-mediated endocytosis, K^+ depletion and chlorpromazine, but was insensitive to caveolae inhibitors, filipin, nystatin or methyl-β-cyclodextrin. Following internalization, FC5 was targeted to early endosomes, bypassed late endosomes/lysosomes and remained intact after transcytosis. The transcytosis process was inhibited by agents that affect actin cytoskeleton or intracellular signaling through PI3-kinase. Pretreatment of human brain endothelial cells with wheatgerm agglutinin, sialic acid, α(2,3)-neuraminidase or *Maackia amurensis* agglutinin that recognizes α(2,3)-, but not with *Sambucus nigra* agglutinin that recognizes α(2,6) sialylgalactosyl residues, significantly reduced FC5 transcytosis. FC5 failed to recognize brain endothelial cells-derived lipids, suggesting that it binds luminal α(2,3)-sialoglycoprotein receptor which triggers clathrin-mediated endocytosis. This putative receptor may be a new target for developing brain-targeting drug delivery vectors.

Keywords: blood–brain barrier, glycocalyx, receptor-mediated endocytosis, sialic acid, single domain antibodies, transcytosis.

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The brain capillary endothelium forms a formidable barrier to the entry of drugs into the central nervous system. The tight junctions that seal cerebral endothelial cells (CEC) prevent circulating compounds, including therapeutic drugs, from reaching the brain by the paracellular route. Other unique characteristics of CEC include lack of fenestrations, low number of pinocytic vesicles and an elaborate, highly negatively charged glycocalyx on their luminal surface (Begley and Brightman 2003). Further barrier to therapeutic brain delivery is the expression of efflux pumps and high enzymatic activity of CEC (Tamai and Tsuji 2000).

Biologics, including peptides, proteins and oligonucleotides could be delivered to the brain via vesicular transport

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Abbreviations used: AME, adsorptive-mediated endocytosis; BBB, blood–brain barrier; BSA, bovine serum albumin; CEC, cerebral endothelial cells; HCEC, human cerebromicrovascular endothelial cell; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; RME, receptor-mediated endocytosis; sdAb, single-domain antibody; WGA, wheatgerm agglutinin.

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across CEC known as transcytosis (Pardridge 2002a). This is a process that requires a specific or non-specific interaction of a ligand with moieties expressed at the luminal surface of CEC, which triggers internalization of the ligand into endocytic vesicles, their movement through the endothelial cytoplasm and exocytosis at the abluminal side of CEC. Different endocytic pathways have been described in CEC: (i) macropinocytosis, a random pathway of internalization of large proteins, (ii) adsorptive-mediated endocytosis (AME) initiated through non-specific charge-based interactions of drugs/biologics with endothelial surface, and (iii) receptor-mediated endocytosis (RME) triggered by a specific interaction with receptors expressed on CEC (Abbott and Romero 1996; Pardridge 2002a). Both AME and RME have been exploited in designing drug-carrying vectors for delivery across the blood–brain barrier (BBB). For example, cationic cell-penetrating peptides, such as SynB vector family (Drin *et al*. 2002), have the ability to deliver hydrophilic molecules across the BBB via a temperature and energy-dependent AME process (Drin *et al*. 2003). Antibodies specific for brain endothelial antigens that undergo RME and transcytosis across the BBB, most notably anti-transferrin receptor antibody (OX26), have been used to shuttle biologics chemically linked to the antibody or encapsulated into antibody-functionalized carriers (e.g. immunoliposomes) across the BBB in experimental animal models (Pardridge 2002a,b).

To discover new antigen-ligand systems that can be exploited for transvascular brain delivery, we used llama single-domain antibody (sdAb) phage-display library (Tanha *et al*. 2002) for differential antigen selection between human lung and brain microvascular endothelial cells (Muruganandam *et al*. 2002; Tanha *et al*. 2003). sdAbs are V_HH fragments of the heavy chain IgGs, which occur naturally and lack light chain, and are half the size (13 kDa) of a single-chain antibody (scFv) (Tanha *et al*. 2002). Two novel sdAbs, FC5 (GenBank no. AF441486) and FC44 (GenBank no. AF441487), which selectively recognized human cerebromicrovascular endothelial cells (HCEC) and transmigrated across the BBB *in vitro* and *in vivo*, were isolated in these studies (Muruganandam *et al*. 2002; Tanha *et al*. 2003). We have then engineered these sdAbs to enable their conjugation with biologics and carriers (Abulrob *et al*. 2005) and to increase their avidity through multimeric display (Zhang *et al*. 2004; Abulrob *et al*. 2005). sdAbs have several advantages over conventional antibodies as potential transvascular brain delivery vectors, including their small size, low non-specific interactions with tissues expressing high levels of Fc receptors (e.g. liver, spleen) and thus low immunogenicity, and remarkable stability against high temperature, pH, and salts (Muyldermans 2001).

The purpose of this study was to characterize mechanisms of FC5 transmigration across the BBB and its putative receptor on HCEC. The evidence presented suggests that transcytosis of FC5 is dependent on clathrin-coated endocytic vesicles and on the recognition of specific oligosaccharide antigenic epitopes on the luminal surface of HCEC.

Materials and methods

Materials

Cell culture plastics were obtained from Becton Dickinson (Mississauga, ON, USA). Dulbecco's modified Eagle's medium was purchased from Invitrogen (Carlsbad, CA, USA), FBS from HyClone (Logan, UT, USA), human serum from Wisent Inc. (Montreal, QC, Canada), and endothelial cell growth supplement from Collaborative Biomedical Products (Bedford, MA, USA). Antibodies were obtained from the following sources: anti-c-Myc-peroxidase antibody from Roche (Indianapolis, IN, USA), anti-caveolin and anti-clathrin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), FITC-conjugated anti-mouse and Alexa 568 conjugated anti-rabbit secondary antibodies from Molecular Probes (Eugene, OR, USA), Texas-red conjugated transferrin and calcein-AM were purchased from Molecular Probes. Monensin and bisindolyl-maleimide-1 (BIM) were from Calbiochem (San Diego, CA, USA). Optiprep was purchased from Accurate Chemical and Scientific Corp (Westbury, NY, USA). Purified human transferrin receptor and monoclonal anti-CD71 (anti-transferrin receptor) antibody were purchased from Research Diagnostics Inc. (Flanders, NJ, USA). [^{14}C]Sucrose was purchased from Perkin Elmer (Boston, MA, USA). Tetramethylbenzidine/hydrogen peroxide substrate system was procured from R & D systems (Minneapolis, MN, USA). EZ link sulfo-NHS-LC-LC-biotin and bicinchoninic acid assay were purchased from Pierce (Rockford, IL, USA). All other chemicals were from Sigma (St Louis, MO, USA).

FC5 sdAb cloning, expression and purification

FC5 is a variable domain (V_HH) of the llama heavy chain antibody with encoding mRNA and amino acid sequences deposited in the GenBank (no. AF441486 and no. AAL58846, respectively). DNA encoding FC5 was cloned into the *Bbs*I/*Bam*HI sites of plasmid pSJF2 to generate expression vector for FC5 as described previously (Muyldermans 2001; Muruganandam *et al*. 2002). The DNA constructs were confirmed by nucleotide sequencing on 373A DNA Sequencer Stretch (PE Applied Biosystems, Foster City, CA, USA) using primers fdTGIII, 5′-GTGAAAAAATTATTATTATTCGCAATTCCT-3′ and 96GIII, 5′-CCCTCATAGTTAGCGTAACG-3′. The FC5 was expressed in fusion with His_5 and c-myc tags (Muruganandam *et al*. 2002) to allow for purification by immobilized metal affinity chromatography using HiTrap Chelating^{TM} column and for detection by immunochemistry, respectively. Single clones of recombinant antibody-expressing bacteria *Escherichia coli* strain TG1 were used to inoculate 100 mL of M9 medium containing 100 μg/mL of ampicillin, and the culture was shaken overnight at 200 r.p.m. at 37°C. The grown cells (25 mL) were transferred into 1 L of M9 medium (0.2% glucose, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH4CL, 0.05% NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) supplemented with 5 μg/mL of vitamin B1, 0.4% casamino acid, and 100 μg/mL of ampicillin. The cell culture was shaken at 25°C for 24 h at 200 r.p.m and subsequently supplemented with 100 mL of 10 × induction medium Terrific Broth containing 12% Tryptone, 24% yeast extract, and 4% glycerol. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside

(1 mM). After induction, the culture was shaken for an additional 72 h at 25°C, and the periplasmic fraction was extracted by the osmotic shock method (Anand *et al*. 1991). The FC5 fragments were purified by immobilized metal-affinity chromatography using HiTrap Chelating column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). FC5 produced was eluted in 10 mM HEPES buffer, 500 mM NaCl, pH 7.0, with a 10–500-mM imidazole gradient and peak fractions were extensively dialyzed against 10 mM HEPES buffer, 150 mM NaCl, 3.4 mM EDTA, pH 7.4. The molecular weight of FC5 is 13.2 kDa and that of FC5 fusion protein with c-myc and His_5 tags is 15.2 kDa.

To improve the avidity of FC5, a pentameric FC5 (P5) was created by fusing FC5 gene upstream of the D17E/W34A verotoxin B-subunit (VT1B) mutant. Detailed protocols for pentamerization of llama sdAbs on a VT1B scaffold were recently described (Zhang *et al*. 2004).

Cell culture

Primary HCEC cultures were isolated from human temporal cortex removed surgically from perifocal areas of brain affected by idiopathic epilepsy. Cells were dissociated, cultured and characterized as previously described in detail (Stanimirovic *et al*. 1996; Muruganandam *et al*. 1997). The morphological, phenotypic, biochemical and functional characteristics of these HCEC cultures have been described previously (Stanimirovic *et al*. 1996; Muruganandam *et al*. 1997). Passages two to six of HCEC were used for the experiments in this study.

Cell viability in the presence of FC5 and various pharmacological agents was assessed by the vital dye calcein-AM release assay as described previously (Wang *et al*. 1998).

The uptake of FC5 into HCEC was tested 15–90 min after adding 5 µg/mL of FC5 in the absence or presence of various pharmacological modulators of endocytosis. To visualize the intracellular distribution of FC5, cells were fixed, permeabilized and probed with the anti-c-myc antibody (1 : 100; 1 h) followed by incubation with FITC-labeled anti-mouse IgG (1 : 250; 1 h).

Transport across the *in vitro* blood–brain barrier model

HCEC (80 000 cells/membrane) were seeded on a 0.5% gelatin-coated Falcon tissue culture inserts (pore size 1 µm; surface area 0.83 cm^2) in 1 mL of growth medium. The bottom chamber of the insert assembly contained 2 mL of growth medium supplemented with the fetal human astrocyte-conditioned medium in a 1 : 1 (v/v) ratio (Muruganandam *et al*. 1997). The model was virtually impermeable for hydrophilic compounds with molecular weight > 1 kDa (Muruganandam *et al*. 1997).

Transport studies were performed 7 days post-seeding as described previously (Muruganandam *et al*. 1997, 2002). Filter inserts were rinsed with transport buffer [phosphate buffered saline (PBS) containing 5 mM glucose, 5 mM $MgCl_2$, 10 mM HEPES, 0.05% bovine serum albumin (BSA), pH 7.4] and allowed to equilibrate at 37°C for 30 min. Experiments were initiated by adding 10 µg/mL FC5 to either the apical or basolateral side of inserts containing either 0.5% gelatin-coated inserts without cells, control HCEC or HCEC pre-exposed to various pharmacological modulators for 30 min. Transport studies were conducted at 37°C with plates positioned on a rotating platform stirring at 30–40 r.p.m. Aliquots (100 µL) were collected from the opposite chamber at various time intervals (5, 15, 30, 60, 90 min) and replaced with fresh buffer. The amount of FC5 transported across empty inserts or HCEC monolayers was determined by enzyme linked immunosorbent assay (ELISA) (see below). To control for HCEC membrane integrity and to estimate paracellular diffusion, the apical-to-basolateral and basolateral-to-apical clearance rates of [^{14}C]sucrose were determined and calculated essentially as described previously (Muruganandam *et al*. 2002; Garberg *et al*. 2005) across the same monolayers used for FC5 transport studies. Sample-associated radioactivity in 50-µL aliquots was measured using a Mircobeta Trilux liquid scintillation counter (Wallac, Turku, Finland).

Clearance was calculated as $Cl\ (ml) = C_A/C_I \times V_A$, where C_I is the initial tracer or sdAb concentration in the donor chamber, C_A is the tracer or sdAb concentration in the acceptor chamber, and V_A is the volume of the acceptor chamber. Clearance of FC5 was linear between 15 and 60 min, while saturation was reached between 60 and 90 min (Muruganandam *et al*. 2002). The effects of pharmacological agents on FC5 transmigration was subsequently assessed at 30 min. HCEC monolayer is virtually impermeable for non-selected sdAbs isolated from the same library or fluorescent dextran of similar molecular weight (Muruganandam *et al*. 2002).

Laser scanning confocal microscopy

A co-localization of FC5 with clathrin or caveolin-1 was studied by double immunofluorescence labeling. HCEC were first incubated with 5 µg/mL FC5 for 30 min, washed, fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 for 10 min. Cells were then blocked with 4% goat serum for 1 h. After blocking, cells were first incubated with anti-c-Myc monoclonal antibody (1 : 100) for 1 h followed by extensive washing, and then with FITC anti-mouse IgG secondary antibody (1 : 250) for 1 h. After a second overnight blocking with 4% goat serum, HCEC were incubated with either anti-clathrin (1 : 100) or anti-caveolin-1 (1 : 300) polyclonal antibody for 1 h, and then Alexa 568-conjugated anti-rabbit IgG secondary antibody (1 : 300) for 1 h. Texas red-conjugated transferrin (1 µM) and cathepsin B monoclonal antibody (1 : 200) were used as markers for early and late endosomes, respectively. Coverslips with stained cells were washed five times in Hank's balanced salt solution and mounted in fluorescent mounting medium (Dako, Mississauga, ON, Canada).

Imaging of cells processed for double immunochemistry was performed using Zeiss LSM 410 (Carl Zeiss, Thornwood, NY, USA) inverted laser scanning microscope equipped with an Argon\Krypton ion laser and a Plan neofluar 63 ×, 1.3 numerical apparatus oil immersion objective. Confocal images of two fluoroprobes were obtained simultaneously to exclude artifacts from sequential acquisition, using 488 and 568 nm excitation laser lines to detect FITC (BP505-550 emission) and Texas red/Alexa 568 fluorescence (LP590 emission), respectively. All images were collected using the same laser power and pinhole size for the respective channels and processed in identical manner.

Omission of primary antibodies resulted in no staining. No cross-reactivity was observed between the primary and non-corresponding secondary antibodies.

Cellular fractionation

To isolate protein and lipid fractions, HCEC were washed with PBS, scraped and lyophilized. Cell remnants were dissolved in 50 mM

Tris, pH 7.2. Proteins were separated from lipids with a chloroform–methanol mixture using a modified version of the protocol of Wessel and Flugge (1984). Before drying the lipid fraction under a stream of nitrogen gas, galactosylceramide was added as a positive control. Proteins and lipids were dissolved in 6 M urea and methanol, respectively.

Detergent-free method was used to isolate low-density membrane fraction as described previously (Abulrob *et al.* 2004). All steps were carried out at 4°C and all buffers were supplemented with a cocktail of protease inhibitors (Sigma). Plasma membrane fractions were prepared from five 75-cm^2 tissue culture flasks of confluent HCEC incubated in the presence of 5 μg/mL FC5 for 30 min. Each flask was washed twice with 10 mL of buffer A (0.25 M sucrose, 1 mM EDTA, and 20 mM Tricine, pH 7.8), cells were then collected by scraping in 5 mL buffer A, pelleted by centrifugation at 1400 *g* for 5 min (Beckman J-68), re-suspended in 1 mL of buffer A, and homogenized by 20 up/down strokes with a Teflon glass homogenizer. Homogenized cells were centrifuged twice at 1000 *g* for 10 min (Eppendorf Centrifuge 5415C), and the two post-nuclear supernatant fractions were collected, pooled, overlayed on top of 23 mL of 30% Percoll solution in buffer A and ultracentrifuged at 83 000 *g* for 30 min in a Beckman 60Ti. The pellet, representing plasma membrane fraction, was collected and sonicated six times at 50J/W per s (Fisher Sonic Dismembrator 300). The sonicated plasma membrane fraction was mixed with 50% Optiprep in buffer B (0.25 M sucrose, 6 mM EDTA, and 120 mM Tricine, pH 7.8) (final Optiprep concentration, 23%). The entire solution was placed at the bottom of the Beckman SW41Ti tube, overlayed with a linear 20–10% Optiprep gradient, and centrifuged at 52 000 *g* for 90 min using SW41Ti (Beckman Instruments). The top 5 mL of the gradient was collected and mixed with 50% Optiprep in buffer B, placed on the bottom of a SW41Ti tube, overlayed with 2 mL of 5% Optiprep in buffer A and centrifuged at 52 000 *g* for 90 min. An opaque band located just above the 5% interface was designated the 'caveolae fraction.' The gradient was fractionated into 1.25-mL fractions.

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and western immunoblot analysis

For immunoblot detection of FC5, caveolin-1 and clathrin heavy chain proteins, each fraction of the final Optiprep gradient was resolved on SDS–PAGE under reducing conditions. The separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Nepean, ON, Canada). After blocking with 5% skim milk for 1 h, the membrane was probed with HRP-conjugated anti-c-Myc monoclonal antibody (dilution 1: 1000), polyclonal anti-caveolin antibody (dilution 1 : 500) or anti-clathrin antibody (dilution 1 : 500) in TBS-Tween with 5% skim milk for 2 h. ECL plus western blotting detection system was used to detect signals.

In another experiment, western blot analysis was used to determine whether FC5 recognizes purified human transferrin receptor. Human placental transferrin receptor was electrophoresed in 10% SDS–PAGE under reducing conditions, electrophoretically transferred onto a polyvinylidene difluoride membrane and probed with either the anti-transferrin receptor antibody CD71 (1 : 500) or pentameric FC5 construct (P5) 1 : 100 for 2 h as the primary antibodies. Peroxidase anti-mouse IgG (1 : 5000) and peroxidase labeled anti-c-Myc monoclonal antibody (1 : 5000) were used as secondary antibodies for CD71 mAb and P5 sdAb, respectively.

Enzyme-linked immunosorbent assay (ELISA)

To measure the amount of FC5 transmigrated across the *in vitro* BBB model, 50 μL aliquots collected from the appropriate compartment were immobilized overnight at room temperature in a HisGrab nickel coated 96-well plate (Pierce). After blocking the plates with 2% BSA for 2 h at room temperature, anti-c-Myc monoclonal antibody conjugated to HRP was added at a dilution of 1 : 5000 for 1 h. After washing, the bound FC5 was detected with tetramethylbenzidine/hydrogen peroxide substrate system. The signal was measured at 450 nm on a microtiter plate reader. FC5 concentrations in collected aliquots were determined from a standard curve constructed using known FC5 concentrations.

To measure FC5 binding to HCEC protein and lipid fractions, isolated fractions were coated onto a flexible 96-well ELISA plate by drying overnight at 37°C. The ELISA plate was blocked with 0.5% BSA in PBS for 2 h. Plates were then incubated with either FC5 antibody or with the O1 antibody against galactosylceramide (kind gift from Dr J. Totter, University of Heidelberg, Germany). The FC5 antibody was detected with the mouse anti-myc antibody 9E10. The assay was further carried out as described (Roovers *et al.* 1998).

Results

FC5 transmigration across HCEC is polarized and charge independent

FC5 was not toxic to HCEC even at very high concentrations (1 mg/mL). The permeability of [^{14}C]sucrose across the *in vitro* BBB model was not significantly different in the absence or presence of 10 μg/mL FC5 [$P_e = (0.897 \pm 0.11) \times 10^{-3}$ and $(0.862 \pm 0.18) \times 10^{-3}$ cm/min, respectively,], suggesting that FC5 does not affect the paracellular permeability of HCEC.

Transcytosis of FC5 across the *in vitro* BBB model was polarized: 12-fold higher transport of FC5 from apical-to-basolateral than from basolateral-to-apical chamber was observed in only 30 min **(**Fig. 1a**)**. In contrast, [^{14}C]sucrose, a marker for paracellular diffusion, exhibited expected equal (i.e. non-polarized) distribution from apical-to-basolateral and from basolateral-to-apical side of the cellular monolayer (Fig. 1a).

To investigate whether FC5 is internalized and transported by macropinocytosis, FC5 transmigration was tested in the presence of 500 μM amiloride, a compound that inhibits the formation of macropinosomes without affecting coated pits-mediated endocytosis (West *et al.* 1989). Amiloride had no effect on transendothelial migration of FC5 (Fig. 1b).

The contribution of AME to FC5 transcytosis was assessed because sdAbs are positively charged (the calculated isoelectric point of FC5 is ∼9.23). HCEC were pre-incubated for 30 min with highly cationic protamine sulfate (40 μg/mL) or poly-L-lysine (300 μM), both previously

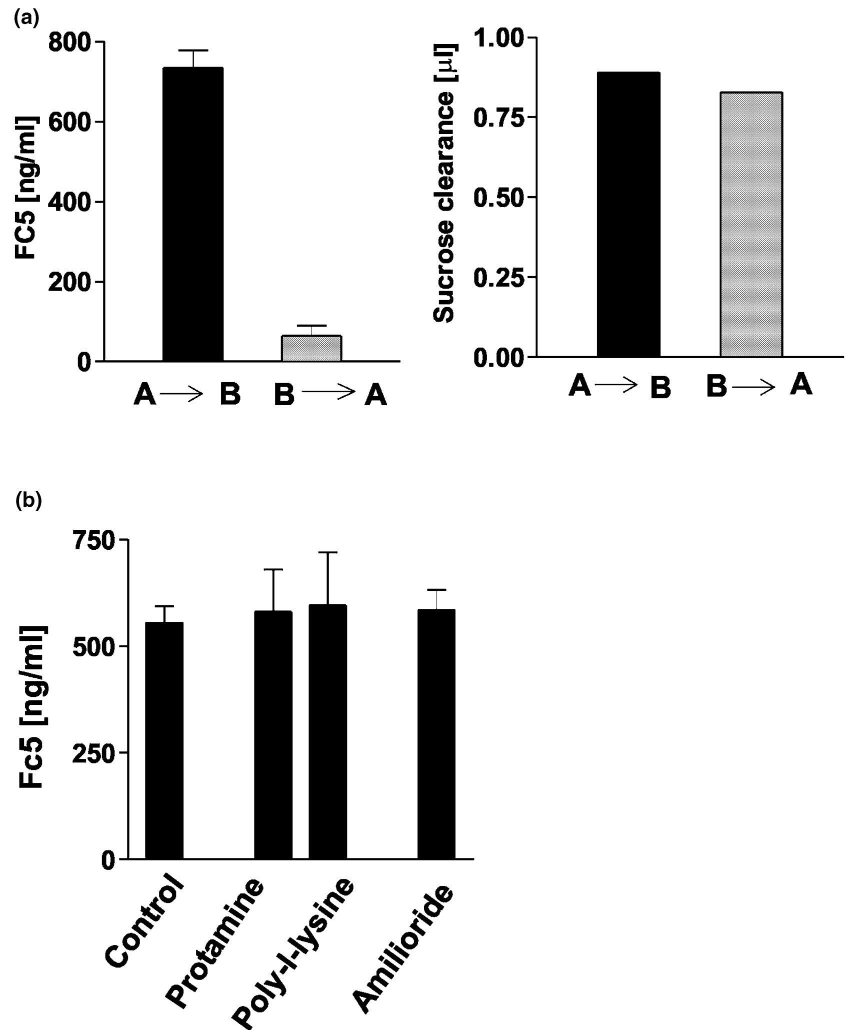


Fig. 1 (a) Polarized transmigration of FC5 across *in vitro* blood–brain barrier (BBB) model. Transport studies were initiated by adding 10 µg/mL FC5 to either apical (A → B) or basolateral (B → A) compartment and the amount of FC5 in the opposite compartment was determined after 30 min as described in Materials and methods. [^{14}C]Sucrose distribution across the same HCEC monolayers was used as internal control for paracellular transport. (b) Effects of pharmacological inhibitors of adsorptive-mediated endocytosis (AME) and macropinocytosis on transmigration of FC5 across *in vitro* BBB model. HCEC were pretreated for 30 min with AME inhibitors, protamine sulfate (40 µg/mL) and poly L-lysine (300 µM), or micropinocytosis inhibitor, amiloride (500 µM), and FC5 transport was measured over 30 min as described in Materials and methods. Each bar represents mean ± SD from six replicate membranes.

shown to inhibit AME (Sai *et al.* 1998) prior to assessing FC5 uptake and transport. Neither compound affected FC5 uptake into HCEC (data not shown) nor transport across the *in vitro* BBB model (Fig. 1b), suggesting that FC5 binding to and transmigration across HCEC is charge independent.

Surprisingly, wheatgerm agglutinin (WGA), tested in these studies for its reported ability to stimulate AME in BBB (Banks *et al.* 1998), significantly inhibited FC5 transmigration, providing initial evidence that endothelial glycocalyx might participate in this process through mechanisms other than charge-mediated interactions. This possibility was further explored in studies described later.

FC5 transport across HCEC is energy dependent

To investigate the energy dependence of FC5 trancytosis, uptake and transport of FC5 were measured at 37°C and at 4°C. Intracellular FC5 was detected by immunochemistry for c-myc followed by FITC-labeled secondary antibody. FC5 was internalized into HCEC as early as 15 min (not shown) and was detected in a majority of cells 30 min after addition at 37°C (Fig. 2a). Marked reductions of both intracellular accumulation (Figs 2a and b) and *trans*-endothelial migration (Fig. 2c) of FC5 were observed at 4°C compared with 37°C. The transport of [^{14}C]sucrose across the BBB model was not affected by temperature (data not shown). A simultaneous inhibition of respiration and the glycolytic pathway by exposing HCEC to 5 mM sodium azide (NaN_3) and 5 mM 2-deoxyglucose for 30 min in a glucose-free medium resulted in a near-complete inhibition of FC5 transmigration (Fig. 2c). This treatment has been shown to result in a complete depletion of cellular ATP in other cell types (Ronner *et al.* 1999). Pretreatment of HCEC with the Na^+,K^+-ATPase pump inhibitor, ouabain (1 µM) for 30 min also reduced FC5 transport across HCEC by 40% (Fig. 2d).

FC5 transcytosis occurs via clathrin-coated vesicles

Two major energy-dependent receptor-mediated endocytosis/transcytosis routes for FC5 transmigration, clathrin-coated vesicles and caveolae, were investigated using co-localization studies and endocytosis inhibitors.

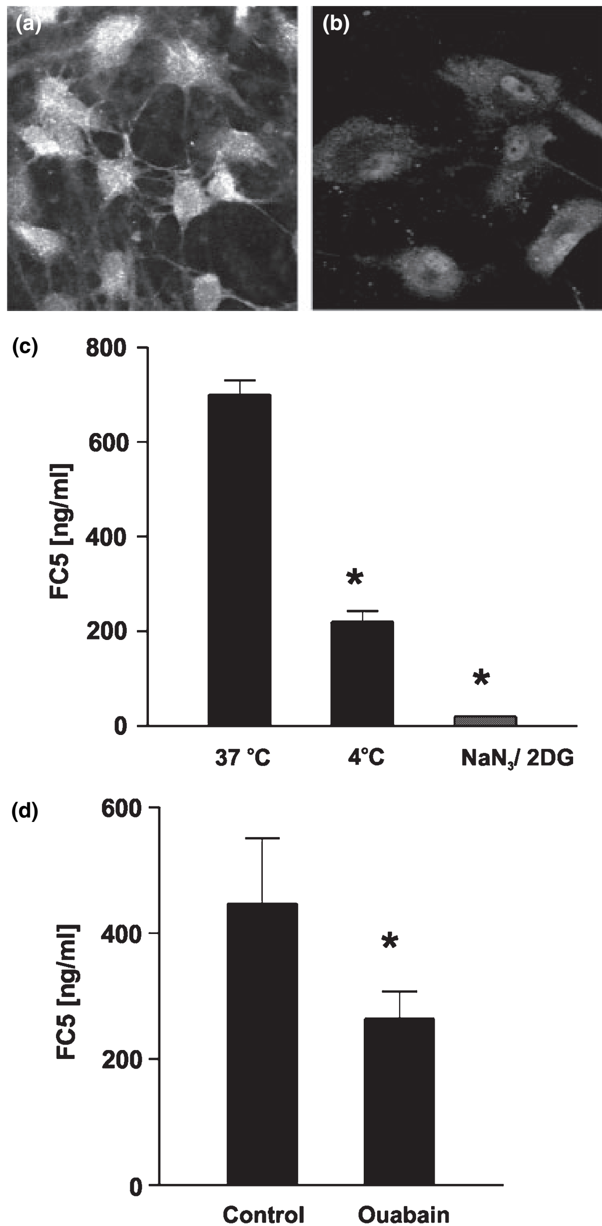


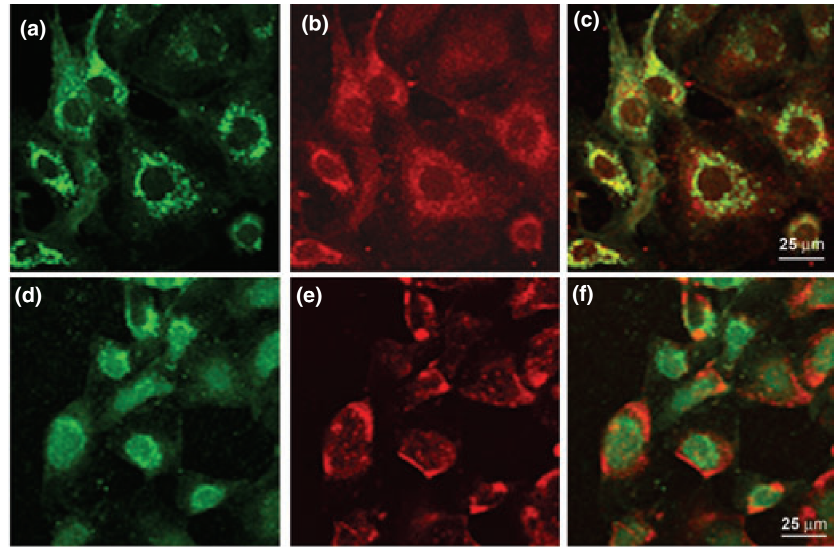
Fig. 2 Energy-dependence of FC5 uptake into HCEC and transmigration across *in vitro* blood–brain barrier model. Confocal microscopy images of FC5 uptake into HCEC at 37°C (a) and at 4°C (b). Cells were exposed to 5 μg/mL FC5 for 30 min and processed for double immunochemistry for c-myc tag of FC5 as described in Materials and methods. (c) Transcellular migration of 10 μg/mL FC5 across HCEC at 37°C or 4°C, or after a 30-min exposure of HCEC to 5 mM NaN_3 and 5 mM deoxyglucose (2DG) for 20 min in glucose-free medium. FC5 transmigration was determined 30 min after addition to HCEC as described in Materials and methods. (d) The effect of Na^+,K^+-ATPase inhibitor, ouabain, on transcellular migration of FC5 across HCEC. Cells were pretreated with 1 μM ouabain for 30 min and FC5 transport was measured over 30 min as described in Materials and methods. Each bar represents mean ± SD. from six replicate membranes. *Significant differences ($p < 0.05$; Student's *t*-test) from 37°C or untreated cells.

Double immunocytochemistry for caveolin-1 and FC5 in HCEC exposed to 5 μg/mL FC5 for 30 min showed no co-localization of caveolin-1 immunofluorescence (red) with FC5 immunofluorescence (green) (Figs 3d–f). In contrast, clathrin immunofluorescence (red) mostly co-localized with that of FC5 (green) (Figs 3a–c). Furthermore, after HCEC fractionation by the density gradient centrifugation, FC5 immunoreactivity on western blot appeared in the same fractions (nos 7, 8 and 9) as did clathrin immunoreactivity, but was absent from caveolin-1 enriched fractions (nos 2 and 3) (Fig. 3g).

Uptake and transmigration of FC5 was examined in cells pretreated for 30 min with pharmacological inhibitors of clathrin-mediated endocytosis, including chlorpromazine (50 μg/mL) and a hypotonic K^+ depletion buffer (0.14 M NaCl, 2 mM $CaCl_2$, 1 mg/mL glucose, 20 mM HEPES, pH 7.4 diluted 1 : 1 with water) or inhibitors of caveolae-mediated endocytosis including filipin (5 μg/mL), nystatin (5 μg/mL) and methyl-β cyclodextrin (5 mM). Chlorpromazine disrupts the recycling of AP-2 from endosomes and prevents the assembly of coated pits on the plasma membrane (Subtil *et al*. 1994) whereas K^+ depletion arrests clathrin-coated vesicle formation (Hansen *et al*. 1993). Filipin and nystatin bind cholesterol (Schnitzer *et al*. 1994), while methyl-β cyclodextrin extracts cholesterol from plasma membrane, resulting in disruption of cholesterol-rich caveolae vesicles (Gumbleton *et al*. 2000). None of the caveolae-mediated endocytosis inhibitors tested affected the transmigration of FC5 across *in vitro* BBB model (Fig. 3h). In contrast, chlorpromazine and K^+ depletion inhibited the transmigration of FC5 by 52 and 46%, respectively (Fig. 3h).

To investigate intracellular fate of FC5 after endocytosis, co-localization studies were performed with markers of early and late endosomes/lysosomes. FC5 co-localized with the early endosome marker, texas red-conjugated transferrin (Figs 4a–c) (Sonnichsen *et al*. 2000), but did not co-localize with cathepsin B (Figs 4d–f), a marker for late endosomes (Roshy *et al*. 2003). Transcytosed FC5 collected from the basolateral chamber of the BBB model was indistinguishable from FC5 added to the apical compartment on a western blot (Fig. 4g), indicating that FC5 bypasses lysosomes and remains intact during transcytosis across HCEC. Unselected sdAbs from the same library could not be detected in the basolateral chamber of the model (Muruganandam *et al*. 1997) indicating that FC5 does not pass into the basolateral chamber via paracellular transport.

Transport of FC5 was also sensitive to neutralization of intracellular compartments by the cationic ionophore monensin. Monensin breaks down Na^+ and H^+ gradients in endosomal and lysosomal compartments, raising the pH of endocytic vesicles from 5.5 to greater than 7 and therefore inhibiting receptor recycling (Basu *et al*. 1981; Tycko *et al*. 1983). Monensin (25 μM) inhibited FC5 transcytosis across HCEC by 34% (Fig. 4h), demonstrating that acidified



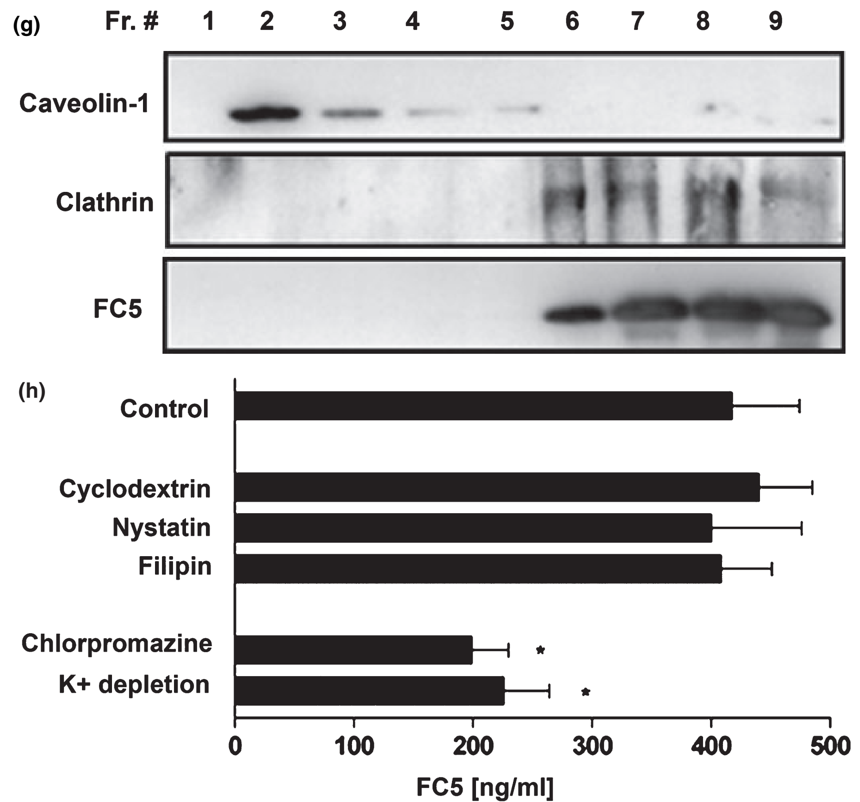


Fig. 3 Role of clathrin-coated pits and caveolae in endocytosis and transcytosis of FC5 in HCEC. Co-localization of FC5 [green fluorescence; (a)] and clathrin [red fluorescence; (b)] in HCEC cells. Overlay image is shown in (c). Co-localization of FC5 [green fluorescence; (d)] and caveolin-1 [red fluorescence; (e)]. Overlay image is shown in (f). Cells were exposed to FC5 for 30 min, washed and processed for double immunocytochemistry as described in Materials and methods. Images are representative of three to five separate experiments. (g) Western blots showing distribution of caveolin-1, FC5, and clathrin heavy chain immunoreactivity in subcellular fractions obtained from HCEC exposed to FC5 for 30 min. HCEC cells were fractionated as described in Materials and methods. Western blots are representative of three separate experiments. (h) Effects of pharmacological inhibitors of caveolae-mediated endocytosis, methyl-β-cyclodextrin (5 mM), nystatin (5 μg/mL) and filipin (5 μg/mL), or inhibitors of clathrin-coated pits-mediated endocytosis, chlorpromazine (50 μg/mL) or potassium-free buffer on transmigration of FC5 across *in vitro* BBB model. Human CEC were pretreated for 30 min with above inhibitors and FC5 transport was measured over 30 min as described in Materials and methods. Each bar represents mean ± SD from six replicate membranes. *Significant differences ($p < 0.05$; one-way ANOVA, followed by Dunnett's multiple comparison between means).

intracellular compartments and recycling of the FC5 putative receptor might be important for maintenance of efficient transendothelial transport.

Signaling pathways involved in FC5 endocytosis/transcytosis in HCEC

To determine requirement for cytoskeletal machinery in transcytosis of FC5, HCEC were pre-incubated for 30 min with the actin depolymerizing agents, cytochalasin D (0.5 μM) or latrunculin A (0.1 μM) (Rotsch and Radmacher 2000), or with the microtubule disrupting agents, nocodazole (20 μM) (Bayer *et al*. 1998) or colchicine (20 μM) (Liu *et al*. 1993). Both cytochalasin D and latrunculin A substantially (70–80%) reduced apical to basolateral transport of FC5 across HCEC (Fig. 5a). In contrast, microtubule-disrupting agents did not interfere with FC5 transcytosis (Fig. 5a).

To determine which signaling pathways modulate transcytosis of FC5, HCEC were pre-incubated for 30 min with

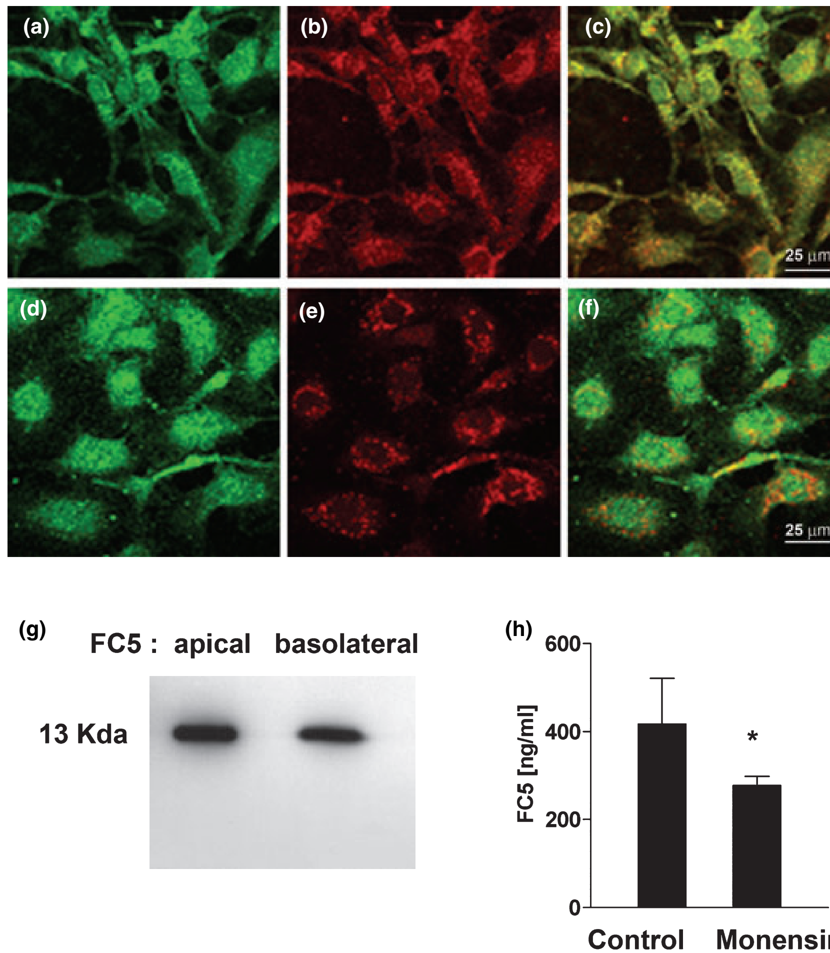


Fig. 4 FC5 processing in endosomes. Co-localization of FC5 [green fluorescence; (a)] and Texas red-conjugated tranferrin [red fluorescence; (b)] in HCEC cells. Overlay image is shown in (c). Co-localization of internalized FC5 [green fluorescence; (d)] and cathepsin-B [red fluorescence; (e)] in HCEC cells. Overlay image is shown in (f). CEC are processed for immunochemistry and confocal microscopy as described in Materials and methods. (g) Western blot of FC5 prior to (top) and after (bottom) transcytosis across HCEC *in vitro* BBB model. (h) Transcellular migration of 10 μg/mL FC5 across HCEC pretreated with 25 μM monensin for 30 min. Transport studies were performed as described in Materials and methods.

one of the following modulators: tyrosine kinase inhibitor, genistein (50 μM); protein kinase C (PKC) inhibitor, bisindolyl-maleimide-1 (BIM-1; 5 μM); PI3-kinase inhibitor, wortmannin (0.5 μM); and protein kinase A (PKA) activator, dibutyryl-cAMP (db-cAMP; 500 μM). FC5 transcytosis across HCEC was not affected by either genistein (Fig. 5b) or db-cAMP (Fig. 5b), was reduced by 25% in the presence of PKC inhibitor (Fig. 5b) and was almost completely blocked by PI3 kinase inhibitor (Fig. 5b). None of the pharmacological agents used was toxic to the cells (data not shown).

Role of carbohydrate epitope(s) in FC5 transcytosis

The role of endothelial glycocalyx in FC5 transcytosis was indicated by the observation that WGA, a lectin known to stimulate AME in BBB (Banks *et al.* 1998), inhibited FC5 uptake (Figs 6a and b) into HCEC.

To test whether proteoglycans, glycoproteins which carry large unbranched polymers composed of 20–200 repeating disaccharide units of sulfated glycosaminoglycan chains (Bandtlow and Zimmermann 2000) and are abundantly expressed in CEC (Floris *et al.* 2003), mediate FC5 transcytosis across HCEC competition experiments with several known soluble glycosaminoglycans found on membranes were performed. Pre-incubation of HCEC with heparin sulfate (50 U/mL), chondroitin sulfate A (10 μg/mL) and chondroitin sulfate C (10 μg/mL) did not affect FC5 transcytosis across the BBB *in vitro* (data not shown). Similarly, mannan (1 mg/mL) and mannose (50 μM) did not affect FC5 transmigration (data not shown), suggesting that mannose 6–phosphate/insulin-like growth factor 2 receptor, a multifunctional transmembrane glycoprotein involved in BBB transport in developing brain (Urayama *et al.* 2004), was not involved in FC5 internalization.

Because WGA is known to interact with a broad range of sialoconjugates (Parillo *et al.* 2003), the importance of sialic acid residues for endo- and transcytosis of FC5 was examined next. HCEC were pretreated with 200 μM sialic acid, or 0.1–0.2 U of neuraminidase from *Vibrio cholerae* which sheds all sialic acid from a variety of plasma membrane glycoproteins (Suzuki *et al.* 2002), or α(2,3) neuraminidase from *Salmonella typhi*, that is selective for α(2,3)-linked sialic acid. Both FC5 uptake (Figs 6c and d) and its transcytosis across HCEC (Fig. 6e) were inhibited by

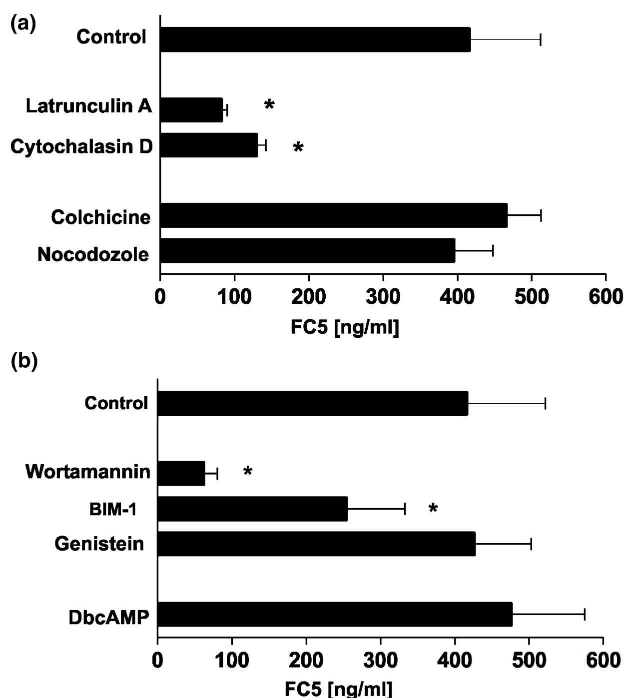


Fig. 5 (a) Role of cytoskeletal network in FC5 transcytosis across HCEC. HCEC were pretreated for 30 min with the actin microfilament inhibitors cytochalasin D (0.5 μM) or latrunculin A (0.1 μM), or with the microtubule inhibitors nocodazole (20 μM) or colchicine (20 μM), and FC5 tranmigration across *in vitro* BBB model was measured over 30 min as described in Materials and methods. (b) Signaling pathway modulators wortmannin (0.5 μM), BIM-1 (5 μM), genistein (50 μM) or dbcAMP (500 mM) were added to HCEC 30 min before addition of 10 μg/mL FC5, and transcytosis across *in vitro* BBB model was measured after 30 min. Each bar represents mean ± SD from six replicate membranes. *Significant differences ($p < 0.05$; one-way ANOVA, followed by Dunnett's multiple comparison between means) from control.

sialic acid and neuraminidase (sialidase). Neuraminidase was especially effective as it reduced FC5 transcytosis by 91% (Fig. 6e). These studies imply that sialic acid is an essential component of the antigenic epitope on HCEC recognized by FC5, as its removal or competition for FC5 binding by exogenous sialic acid interfered with both the uptake and transcytosis of FC5.

The nature of sialoglycoconjugates involved in FC5 transcytosis was examined further by pretreating cells with three sialic acid-binding lectins: wheatgerm agglutinn (WGA; 100 μg/mL) that interacts with a broad range of sialoconjugates, *Sambucus nigra* agglutinin (SNA; 100 μg/mL) and *Maackia amurensis* agglutinin (MAA; 100 μg/mL) that recognize α(2,6) and α(2,3) sialylgalactosyl residues, respectively. WGA and MAA inhibited FC5 transcytosis by 40–50% (Fig. 6f), whereas SNA was ineffective (Fig. 6f).

To investigate whether FC5-recognized sialic acid residues are attached to a glycolipid (ganglioside), HCEC cells were

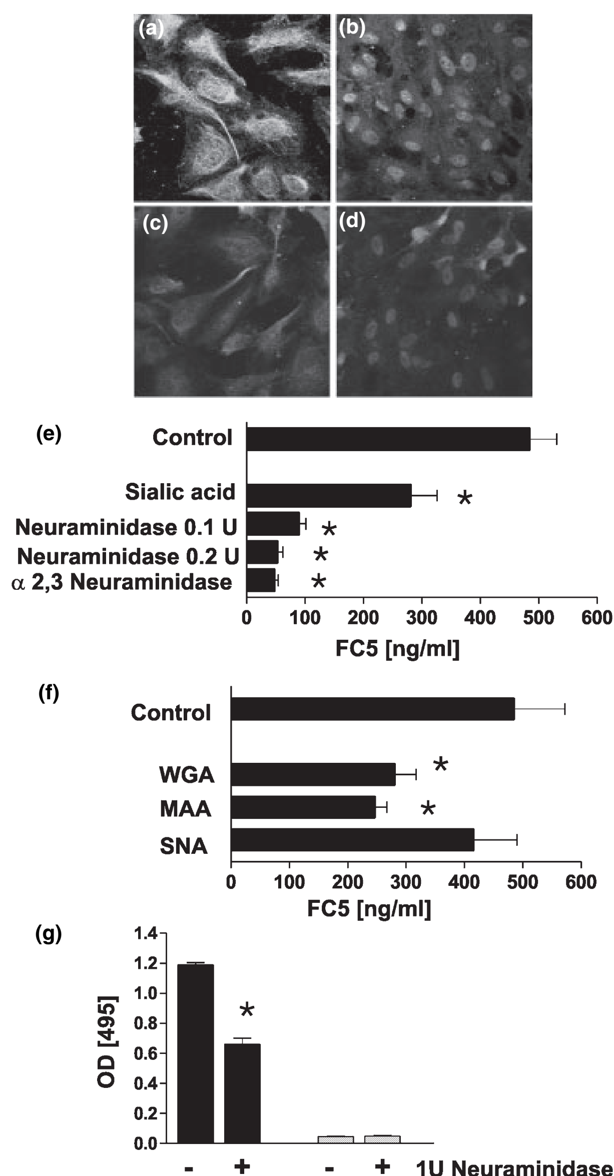


Fig. 6 Role of oligossacharide antigenic epitopes in FC5 uptake into and transcytosis across HCEC. (a–d) Fluorescent micrographs of FC5 uptake in HCEC in the absence (a) or presence of 100 μg/mL WGA (b), 200 μM sialic acid (c) or 0.1 U neuraminidase (d). Uptake was measured over 30 min. (e) Transcytosis of 10 μg/mL FC5 across HCEC pretreated with 200 μM sialic acid or indicated concentrations of neuraminidase for 30 min. (f) Transcytosis of 10 μg/mL FC5 across HCEC pretreated with 100 μg/mL WGA, 100 μg/mL S*ambucus nigra* agglutinin (SNA) or 100 μg/mL *Maackia amurensis* agglutinin (MAA) for 30 min. Transport studies were performed as described in Materials and methods. (g) FC5 binding to isolated protein (black bars) and lipid (gray bars) fractions of HCEC determined by ELISA. Prior to fractionation, lysed cells were incubated in the absence or presence of 1 U/mL neuraminidase for 1 h at 37°C. ELISA on isolated protein and lipid fractions was performed as described in Materials and methods. Each bar represents mean ± SD from six replicates. *Significant differences ($p < 0.05$; one-way ANOVA, followed by Dunnett's multiple comparison between means) from control.

fractionated into protein and lipid fractions as described (Wessel and Flugge 1984). FC5 binding to these fractions in the absence or presence of neuraminidase was examined by ELISA. FC5 binding to the HCEC lipid fraction was negligible (Fig. 6g). FC5 also failed to recognize isolated brain gangliosides (data not shown). In contrast, strong FC5 binding to the HCEC protein fraction was reduced by 50% in the protein fraction of cell lysates exposed to neuraminidase (Fig. 6g). FC5 did not bind to either the protein or lipid fraction of HEK293 cells (data not shown). Galactosylceramide used as a positive control rendered a strong signal for the lipid fraction detected by O1 anti-galactosylceramide antibody (data not shown).

Exclusion of the transferrin receptor

Because transferrin receptors are enriched in CEC (Jefferies *et al*. 1984), are involved in transcytosis across the BBB (Qian *et al*. 2002), and are highly glycosylated (Hayes *et al*. 1992), we investigated whether the putative receptor for FC5 is actually the human transferrin receptor. FC5 and its higher avidity pentameric construct P5 (Abulrob *et al*. 2005) did not bind to immobilized human transferrin receptor in the ELISA assay (Fig. 7a) nor did they recognize the protein on a western blot (Fig. 7b), in contrast to anti-transferrin receptor antibody CD71 (Figs 7a and b). In addition, FC5 uptake (data not shown) and transendothelial transport (Fig. 7c) were not reduced in the presence of a 100-fold excess of holo-transferrin.

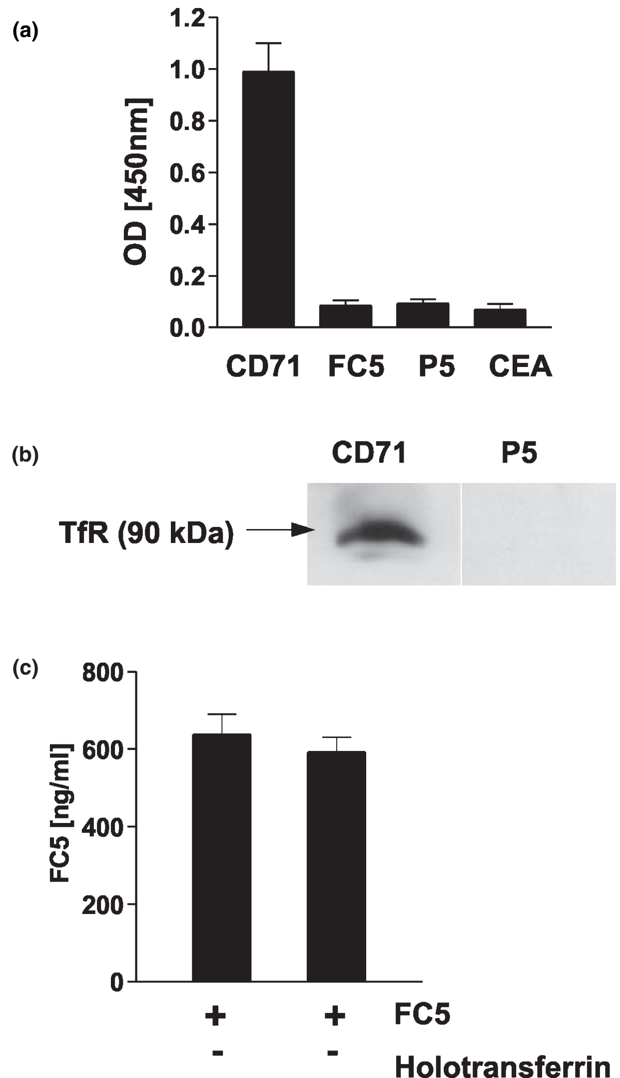


Fig. 7 Lack of transferrin receptor involvement in FC5 transcytosis across *in vitro* BBB. (a) Binding of the anti-transferrin receptor monoclonal antibody, CD71, FC5, pentameric construct of FC5 (P5) or non-related antibody from the same library that recognizes carbohydrate antigen, CEA, to human tranferrin receptor immobilized onto ELISA plate. The plates were read at 450 nm with an automated microplate reader. (b) Western blot of human transferrin receptor immunodetected by anti-CD71, but not by P5. (c) Transcytosis of 10 μg/mL FC5 alone or in the presence of 100-fold (1 mg/mL) of holotransferrin across HCEC monolayers. Transport was measured over 30 min as described in Materials and methods. Each bar represents mean ± SD from six replicate determinations.

Discussion

Single-domain antibodies (sdAbs), sometimes also called 'nanobodies', are the V_HH fragments of the heavy chain IgGs, which occur naturally in camelid species and lack light chain (Muyldermans 2001). Because of their small size (13 kDa), sdAbs often recognize unusual and hidden antigens that cannot be accessed by full-size IgGs such as epitopes in ion channel cavities (Muyldermans 2001) or highly glycosylated proteins (Zhang *et al*. 2004, 2005). sdAbs can be easily engineered and expressed, which makes them suitable for creating bi-functional proteins or bi-specific antibodies (Willuda *et al*. 2001). These characteristics are highly desirable when designing BBB delivery vectors and may provide several advantages over currently used peptide and antibody-based technologies.

A novel single-domain antibody, FC5, that binds brain endothelial cells and transmigrates across the BBB *in vitro* and *in vivo*, was discovered using a differential panning of llama sdAb phage display library against human lung and brain endothelial cells (Muruganandam *et al*. 2002; Tanha *et al*. 2003). FC5 has strong selectivity for the brain endothelium compared with endothelia from peripheral sources including lung and umbilical cord, or to other brain cells, including astrocytes (Muruganandam *et al*. 2002). Antigenic epitope(s) recognized by FC5 are not specific for human CEC, as the antibody also binds to and transmigrates across rat and mouse CEC, and selectively recognizes brain vessels of all three species (Abulrob *et al*. 2005). Preliminary *in vivo* experiments demonstrated that FC5, in contrast to non-selected sdAbs from the same library, could be detected in the capillary-depleted brain tissue 4–6 h after intra-arterial

injection, whether expressed in phage or purified (Muruganandam *et al*. 2002). Further understanding of mechanisms by which FC5 transmigrates across the BBB is essential for designing brain-targeting strategies based on sdAbs.

The collective evidence presented in this study shows that FC5 uptake and transcytosis occur via clathrin-coated vesicles and are dependent on the recognition of neuraminidase-sensitive, α(2,3)-sialo-glycoconjugates. These conclusions were supported by a series of experiments that demonstrated the polarization and temperature and energy dependence of FC5 transmigration and excluded paracellular diffusion, pore formation and macropinocytosis routes. However, contrary to a common assumption, recent studies on a new class of membrane-penetrating peptides that exhibit charge-mediated BBB selectivity showed that, similar to RME, AME can also be temperature and energy dependent (Drin *et al*. 2003). The failure of AME inhibitors that neutralize negative charge on CEC (Sai *et al*. 1998) to reduce transendothelial transport of positively charged FC5 further suggested RME mechanism. Two major vesicular routes of RME, clathrin-coated pits and caveolae were examined next. Clathrin-coated vesicular pathway of FC5 internalization was indicated by strong co-localization of FC5 with clathrin but not with caveolin immunoreactivity in both intact and fractionated HCEC, and by the inhibition of FC5 transcytosis with treatments previously shown to interrupt clathrin-coated vesicle formation (Hansen *et al*. 1993; Subtil *et al*. 1994). Upon internalization, FC5 was targeted to early endosomes, bypassed late endosomes/lysosomes and was exocytosed into the abluminal compartment without significant intracellular degradation.

The vesicular transcellular transport of FC5 was strongly dependent on the intact actin polymerization. Recent studies have identified several proteins, including Abp1p, Pan1p and cortactin (McPherson 2002), that functionally link the actin filament assembly with clathrin-coated vesicle internalization (Qualmann *et al*. 2000; Schafer 2002).

The complexity of signaling events that control trafficking of clathrin-coated vesicles remains difficult to decipher. FC5 transcytosis was essentially blocked by the PI3-kinase inhibitor, wortmannin, while it was little affected by modulators of other signaling pathways, including PKC-, PKA-, and tyrosine kinase inhibitors. Phosphorylation of inositol lipids by PI3-kinase has been implicated in diverse membrane transport events including clathrin-coated pits pathway (Hansen *et al*. 1995). PI3K-C2α has been co-purified with a population of clathrin-coated vesicles (Gaidarov *et al*. 2001), whereas proteins involved in the function of these vesicles, including AP-2 (Beck and Keen 1991) and dynamin (Zheng *et al*. 1996), interact with PI3 kinase. Although PKC (Gekle *et al*. 1997) and PKA (Goretzki and Mueller 1997) have been implicated in internalization of various receptors, neither appears to be generally required for clathrin-mediated endocytosis (Gekle *et al*. 1997; Goretzki and Mueller 1997). Inhibition of the tyrosine kinase activity of some membrane receptors, including the insulin growth factor receptor, previously exploited for RME-mediated brain delivery (Zhang *et al*. 2002), prevents their internalization (Taghon and Sadler 1994). The lack of genistein effect on FC5 transcytosis suggested that the receptor recognized by FC5 is likely not a tyrosine kinase.

The surface of brain endothelial cells is covered by a dense layer of complex carbohydrates that participate in cell–cell communication, pathogen recognition/adhesion and interactions with the extracellular matrix (Pries *et al*. 2000). Studies using various modulators or competitive inhibitors of surface glucoconjugates demonstrated that neuraminidase-sensitive, α(2,3)-sialic acid residues are important for FC5 antigen recognition, FC5 internalization and transcytosis. Sialic acid residues that can be attached to either glycoproteins or gangliosides are abundant in clathrin-coated pits (Tulp *et al*. 1999). The major gangliosides expressed in HCEC are GM3 and sialyl paragloboside (LM1) (Duvar *et al*. 2000; Kanda *et al*. 2004). FC5 failed to bind lipids extracted from HCEC or to recognize any of the major brain gangliosides indicating the glycoprotein nature of the antigen. As sialic acid residues are expressed in many tissues, the selectivity of FC5 for brain endothelial cells is likely conferred by a protein component of the antigenic epitope.

The transferrin receptor is brain endothelium-enriched (Jefferies *et al*. 1984), N- and O-glycosylated transmembrane protein with multiple sialic acid residues (Hayes *et al*. 1992) that undergoes a clathrin-coated vesicle-mediated endocytosis (Qian *et al*. 2002). The antibody against the transferrin receptor, OX26, has been used as a vector for brain targeting of biologics and liposomes (Qian *et al*. 2002). FC5 failed to recognize purified human transferrin receptor, and holo-transferrin did not compete with FC5 transcytosis. In agreement with this, desialylated and *N*-deglycosylated transferrin receptor variants have been shown to exhibit the same transferrin binding and internalization properties as the native transferrin receptor (Orberger *et al*. 2001). In addition to the transferrin receptor, other iron-carrying molecules, including melanotransferrin (p97) (Demeule *et al*. 2002) and lactoferrin (Fillebeen *et al*. 1999), as well as other receptors, including insulin receptor (Zhang *et al*. 2002) and a low-density lipoprotein receptor (Dehouck *et al*. 1997), have been identified as potential RME routes for brain delivery. Other studies suggested that receptors specifically up-regulated in pathological conditions, such as TNFα receptor (Osburg *et al*. 2002), undergo RME in brain endothelial cells. These proteins have not been specifically excluded as putative antigens recognized by FC5.

In summary, FC5 is a novel single domain antibody that recognizes α(2,3)-sialoglycoprotein expressed on the luminal surface of brain endothelial cells and undergoes actin- and PI3 kinase-dependent transcytosis via clathrin-coated

vesicles. FC5 and its derivatives engineered to provide linker moieties (Abulrob *et al*. 2005) could be developed into brain-targeting vectors for drugs, biologics and nanocarriers. *In vivo* biodistribution studies (Muruganandam *et al*. 2002) demonstrated a significant FC5 accumulation in the brain and its rapid elimination via kidneys and liver, typical for other biologics of the similar size (Harris and Chess 2003; Chen *et al*. 2004). Therefore, improving FC5 pharmacokinetics by strategies such as PEGylation (Harris and Chess 2003; Chen *et al*. 2004) may be necessary for achieving efficient *in vivo* brain targeting. Nonetheless, BBB-targeting sdAbs combine peptide-like size and high charge-mediated binding to brain endothelium (similar to cell-penetrating Syn-B peptides) (Drin *et al*. 2003) with the recognition of cell-specific antigens that undergo transendothelial transport, similar to 'classical' antibody vectors such as OX26 antibody. Unlike peptides, sdAbs are remarkably resistant to proteases, and, unlike full IgGs (Zhang and Pardridge 2001; Schlachetzki *et al*. 2002), they cannot be exported from the brain via the Fc receptor-mediated efflux system at the BBB. These advantages make sdAbs a versatile alternative to current technologies designed to target drugs and biologics to the brain by exploiting vesicular transendothelial transport.

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