

Single domain antibodies as blood–brain barrier delivery vectors

Abedelnasser Abulrob, Jianbing Zhang, Jamshid Tanha,
Roger MacKenzie, Danica Stanimirovic*

*Institute for Biological Sciences, National Research Council of Canada,
1200 Montreal Rd, Bldg M-54, Ottawa, Ontario, Canada, K1A 0R6*

Abstract. Antibodies against various receptors that undergo transcytosis across brain capillary endothelium including transferrin, insulin growth factor and low-density lipoprotein receptors have been used as vectors to deliver drugs or therapeutic peptides into the brain. In this study, recently discovered llama single domain antibody FC5 that transmigrates across cerebral endothelial cells (CEC) *in vitro* and blood–brain barrier (BBB) *in vivo* was engineered to provide ‘linker’ groups for conjugation with large molecules. Horseradish peroxidase (HRP)-tagged IgG was chemically attached to FC5 through an engineered free cysteine (cysFC5) and HRP-IgG-cysFC5 construct was shown to transmigrate across *in vitro* BBB model. Transmigration of FC5 across CEC was enhanced by pentamerizing FC5. Pentameric FC5 also demonstrated increased binding to microdissected human, mouse and rat brain vessels. © 2005 Elsevier B.V. All rights reserved.

Keywords: Single domain antibody; Blood–brain barrier; Drug delivery; Drug targeting; Brain vessels

1. Introduction

Therapeutic pharmaceuticals entry into the brain is greatly restricted at the blood–brain barrier (BBB) through multiple mechanisms, including high resistance tight junctions expressed on the cerebral endothelial cells (CEC), high enzymatic activity on the endothelial plasma membranes and polarized expression of efflux transporters, including P-glycoprotein and ABCG2 [1]. The BBB allows only selective trafficking of few

* Corresponding author. Tel.: +1 613 993 3730; fax: +1 613 941 4475.

E-mail address: Danica.stanimirovic@nrc-cnrc.gc.ca (D. Stanimirovic).

macromolecules and proteins through receptor-mediated transcytosis, adsorptive endocytosis and non-selective macropinocytosis [2].

Antibodies specific for brain endothelial-associated antigens that undergo transcytosis present an attractive avenue for selective delivery of therapeutic agents to the brain. Various receptors that undergo transcytosis across the BBB, including transferrin, insulin growth factor and low-density lipoprotein receptors are essential for physiological transport of proteins into the brain [1]. Antibodies against these receptors, most notably anti-transferrin receptor antibody (OX-26), have been used to shuttle peptides and large molecules chemically linked to the antibody or encapsulated into functionalized carriers (e.g., immunoliposomes) across the BBB in experimental animal models [3].

To discover new antigen–ligand systems that can be exploited for transvascular brain delivery, we performed subtractive panning of the phage-display library of llama single-domain antibodies (sdAb) [4] against human CEC and lung microvascular endothelial cells [5,6]. sdAbs are the V_H 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) [4]. Two sdAbs, FC5 (GenBank No. AF441486) and FC44 (GenBank No. AF441487), which selectively recognized human CEC and transmigrated across the BBB *in vitro* and *in vivo*, were isolated, sequenced and functionally characterized [5,6]. sdAbs have several advantages over conventional antibodies as potential transvascular brain delivery vectors including small size, low non-specific interactions with tissues expressing high levels of Fc receptors (e.g., liver, spleen), remarkable stability against high temperature, pH, and salts, low immunogenicity, and high levels of expression in *E. coli* [7].

In this study we report FC5 engineering to enable its conjugation with drugs, biologics and carriers and improve its binding properties by multimeric display.

2. Materials and methods

2.1. Cloning and purification of *cysFC5*

To obtain a proof of principle that BBB-targeting sdAb can carry large molecules across the BBB, FC5 was engineered to add additional free cysteine that can be used for conjugation with drugs and carriers. DNA encoding sdAb FC5 was cloned into the BbsI/BamHI sites of plasmid pSJF2 to generate expression vector for monomeric FC5 as described previously [5,6]. *cysFC5* gene was generated from FC5 template by a standard PCR using a forward primer that added a cysteine immediately after the His₅ ‘purification’ tag codons. *cysFC5* gene was subsequently cloned into pSJF2 using standard cloning techniques [8]. The integrity of the cloned construct was confirmed by nucleotide sequencing on 373A DNA Sequencer Stretch (PE Applied Biosystems, Streetsville, ON). *cysFC5* was expressed in bacteria *E. coli* strain TG1 and purified by immobilized metal affinity chromatography (IMAC) as described previously [5,6]. The eluted fractions homogenous for *cysFC5* as judged by SDS-PAGE were pooled and extensively dialyzed against 10 mM HEPES buffer, 150 mM NaCl, 3.4 mM EDTA, pH 7.4. Protein concentrations were determined by the bicinchoninic acid assay (BCA). To assure complete reduction of the engineered free cysteine without compromising the conserved Cys22–Cys92 internal disulfide bonds, the *cysFC5* was

exposed to 50 mM Tris (2-carboxyethyl) phosphine hydrochloride containing 5 mM EDTA in PBS overnight at 4 °C followed by rapid separation on G-25 sephadex columns prior to conjugation. These conditions did not compromise antigen binding activity of cysFC5 determined by intact cellular uptake and transmigration across CEC monolayers.

2.2. Conjugation of HRP-IgG to CysFC5

Cross linking between the horseradish peroxidase (HRP)-tagged mouse IgG and cysFC5 was achieved using sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) as cross linking agent. Sulfo-SMCC builds a bridge between an amine (–NH₂) functional group on the HRP-IgG and a sulfahydryl (–SH) group on the cysFC5 sdAb. First, HRP-IgG was maleimide-activated by incubation with a 10 M excess of sulfo-SMCC solution in PBS for 30 min at room temperature. Maleimide reagent was removed by G-25 sephadex columns (Roche Biochemicals, Indianapolis, IN). Maleimide-activated HRP-IgG was cross linked with reduced cysFC5 by mixing 5:1 molar ratio at room temperature for 1 h.

2.3. Pentamerization of FC5 sdAb

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 [9]. The sequence of the P5 in pVT2 plasmid was confirmed by nucleotide sequencing. P5 was produced in *E. coli* exactly as described [9]. To clear the *E. coli* lysate, the protein sample was centrifuged first at 10,000 rpm for 10 min and then at 12,000 rpm for 40 min at 4 °C. Clear supernatant was loaded onto a Hi-Trap Chelating Affinity Column (Amersham Biosciences, Piscataway, NJ) and purified by IMAC as described above.

2.4. SDS-PAGE and Western immunoblot

SDS-PAGE was carried out under non-reducing conditions with 15% polyacrylamide gels. For Western blotting, the separated proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilin P; Millipore, Nepean, ON). After blocking with 5% skim milk for 1 h, the membrane was probed for 2 h with anti-c-Myc monoclonal antibody conjugated to peroxidase (dilution 1:5000); signal was detected by enhanced chemoluminescence.

2.5. In vitro blood–brain barrier transport

Primary human CEC cultures were isolated, maintained and characterized as previously described [10]. Passages 4–7 of different human CEC isolations were used for experiments in this study. Human CEC were seeded at a density of 80,000 cells onto 1 µm polycarbonate membrane filters dipped in 12-transwell plates. Transport studies were performed 7 days post seeding. Filter inserts were rinsed with transport buffer [PBS containing 5 mM glucose, 5 mM MgCl₂, 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 20 µg of sdAb or sdAb constructs to the apical chamber. Aliquots (100 µl) were collected from the basolateral chamber at various time intervals (5–120 min). Ten and 70

kDa-[¹⁴C] dextrans were used as molecular weight controls for mono- and pentameric FC5, respectively, and were quantified by liquid scintillation counting.

To measure the amount of FC5 or P5 in collected aliquots, 50 µl of aliquots were immobilized and dried overnight at room temperature in a nickel-NTA Hissorb 96-well plate (Nunc MaxiSorp). After blocking with 3% BSA in PBS for 2 h, anti-c-Myc monoclonal antibody tagged with HRP was added at a dilution of 1:5000 and detected with tetramethylbenzidine (TMB)/hydrogen peroxide (H₂O₂) substrate system (R and D Systems, Minneapolis, MN). To determine levels of IgG-HRP, aliquots were immobilized and dried overnight in a regular 96-well plate and quantified using TMB/H₂O₂ substrate system. The signal was measured at 450 nm on a microtiter plate reader. Unknown amounts of sdAb (monomer or pentamer) were determined from a standard curve constructed using known concentrations of respective sdAb protein.

2.6. Laser-capture microdissection of brain microvessels

To determine species specificity of FC5, brain vessels in tissue sections of human, mouse or rat brain were stained with lectins Ulex Europeaus Agglutinin I (UEA I), *Griffonia Simplicifolia* Lectin I-Isolectin B₄ (GSLI-Isolectin B₄) or *Ricinus Communis* Agglutinin I (RCA I), respectively, and captured using laser capture microdissection (LCM) microscopy (Arcturus, Mountain View, CA) as recently described [11,12]. The film was removed from LCM caps, immobilized on a 96-well plate, blocked overnight with 3% BSA in PBS and then incubated with anti-c-Myc monoclonal antibody conjugated to HRP (1:5000) for 2 h. The bound antibody was detected using TMB/H₂O₂ substrate system.

2.7. Immunohistochemistry

To study P5 binding to brain microvessels in situ, brain sections were first stained with vessel-selective fluorescein isothiocyanate (FITC)-labeled lectins as described [11,12]. Sections were then incubated with P5 (1:100) for 1 h, washed, and blocked with 4% goat serum for 1 h. To detect P5, sections were first exposed to anti-c-Myc monoclonal antibody (1:100) for 1 h followed by extensive washing, and then to Alexafluor 568-labeled anti-mouse secondary antibody (1:500) for 1 h. Imaging of the slides was performed using Zeiss LSM 410 (Carl Zeiss, Thornwood, NY) inverted laser scanning microscope. Confocal images were obtained simultaneously to exclude artifacts from sequential acquisitions, using 488 and 568 nm excitation laser lines to detect FITC (BP505–550 emission), and Alexafluor 568 fluorescence (LP590 emission), respectively.

3. Results

3.1. FC5 is capable of carrying large molecules across in vitro BBB

Since sdAbs have no available –SH groups for conjugation with therapeutic moieties, FC5 was engineered to express an additional free cysteine. CysFC5 was then conjugated with mouse HRP-IgG using maleimide activation reaction as shown in Fig. 1A. HRP-IgG or HRP-IgG-cysFC5 uptake into human CEC cultures was determined after exposing cells to either construct for 30 min. A significant cellular uptake of IgG-HRP was seen only

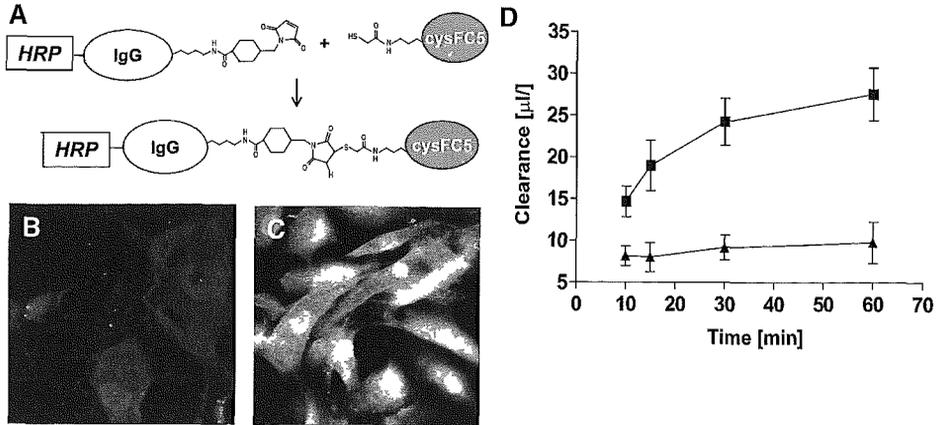


Fig. 1. Conjugation of the blood–brain barrier permeable sdAb FC5 with mouse IgG tagged with horse-radish peroxidase (IgG-HRP) and functional evaluation of the construct in vitro. Additional cysteine moiety was added to FC5 by genetic engineering as described in Materials and methods. (A) cysFC5 was conjugated with maleimide-activated IgG-HRP as in shown reaction. Uptake of IgG-HRP (B) or FC5-IgG-HRP conjugate (C) in human brain endothelial cells in culture. Cells were fixed 30 min after addition of 5 μ g/ml of either conjugate. Uptake was determined in fixed cells using a FITC-labelled anti-mouse secondary antibody (Materials and methods). (D) Transmigration of IgG-HRP (▲) or FC5-IgG-HRP conjugate (■) across the in vitro blood–brain barrier model. Transport studies were performed as described in Materials and methods.

when the molecule was linked to cysFC5 (Fig. 1B and C). Similarly, HRP-IgG linked to cysFC5 exhibited a significant transcellular migration to the abluminal chamber of the in vitro BBB model (Fig. 1D) while transport of IgG-HRP alone across human CEC monolayer was negligible (Fig. 1D).

3.2. Multimeric display of FC5 increases binding to brain vessels

Pentamerization of FC5 on VT1B was performed to introduce avidity in FC5. The construct design is shown in Fig. 2A. The molecular weight (128 kDa) of the purified pentameric construct P5 was confirmed on a Western blot (Fig. 2B). Twenty-six kilo Dalton band (Fig. 2B) corresponds to a single FC5 fused with VT1B. A putative model structure of pentameric sdAb has recently been described [9].

The binding of P5 to LCM-extracted brain vessels from human, mouse and rat was compared to that of either monomeric FC5 or unrelated pentameric sdAb, ES1. P5 sdAb showed higher binding to brain microvessels than monomeric FC5 (Fig. 2C). Unrelated pentameric antibody ES1 showed only marginal binding to immobilized brain vessels (Fig. 2C). Although FC5 was selected by panning against human CEC, it also recognized BBB-selective antigens in brain microvessels of other species, including rat and mouse (Fig. 2C).

Immunohistochemistry using P5 also demonstrated strong vascular staining; P5 immunoreactivity co-localized with vascular structures stained with lectins UEA 1 and RCA 1 in human and rat brain, respectively (Fig. 2D–F). In contrast, P5 failed to stain vessels in rat liver sections (Fig. 2G), indicating that antibody recognizes antigens selectively expressed in brain vessels.

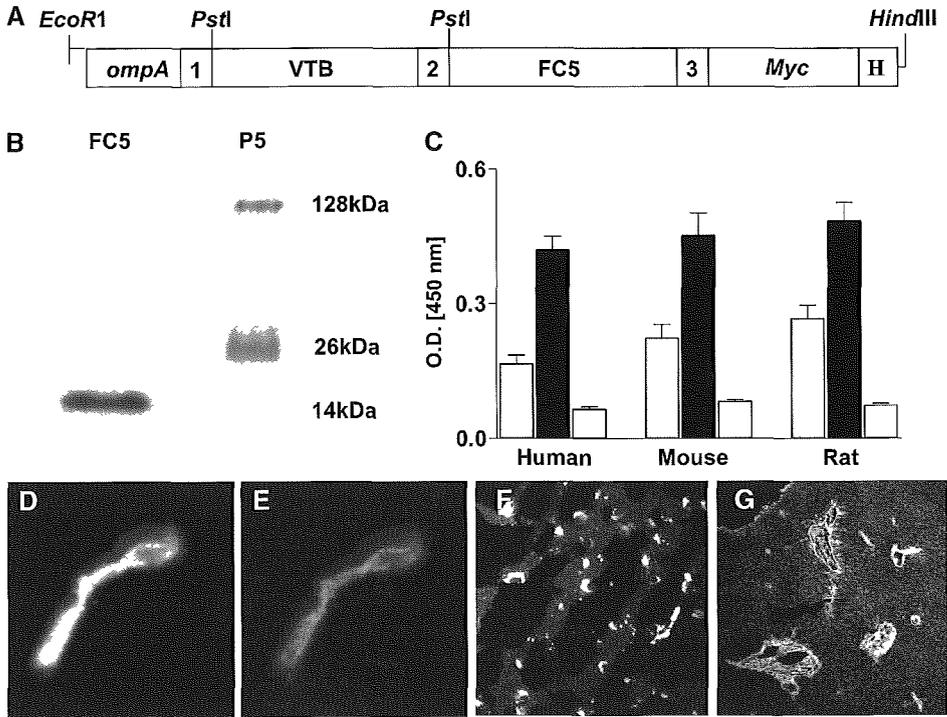


Fig. 2. Construction and functional assessment of pentameric (P5) blood–brain barrier antibody FC5. (A) Schematic of engineered construct of FC5 and verotoxin subunit B. (B) The construct was purified on IMAC and resolved on Western blot; 128 kDa band corresponds to expected molecular weight of P5. (C) Binding of FC5 (gray bar), P5 (black bar) and control pentameric antibody, ES1 (white bar) to LCM extracted and immobilized brain vessels from human, mouse and rat determined by ELISA as described in Materials and methods. Staining of vessels in human brain sections with fluorescein-UEA I (D) and Alexafluor-P5 (E). Staining of vessels in sections of rat brain (F) and liver (G) with RCA I (green) and P5 (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

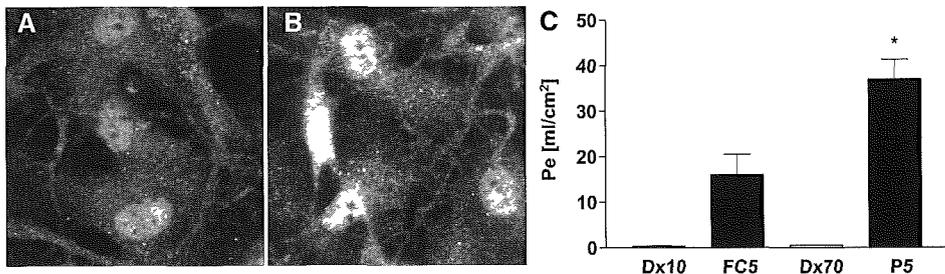


Fig. 3. Uptake of P5 into human CEC at 5 min (A) and 15 min (B) after addition. (C) Transmigration of FC5 and P5 across in vitro blood–brain barrier model; 10 kDa dextran (Dx10) and 70 kDa dextran (Dx70) were used as corresponding molecular weight controls. Transport studies were performed as described in Materials and methods. Each bar is mean±SD for 6 replicate determinations. Asterisks indicates a significant ($p<0.01$; ANOVA) difference from FC5.

3.3. Multimeric display of FC5 increases transport across *in vitro* BBB

The P5 underwent a rapid internalization into cultured human CEC (Fig. 3A and B) within the first 15 min of addition. P5 also demonstrated significantly higher permeability coefficient (P_e) (Fig. 3C) when compared to monomeric FC5 for its ability to transmigrate across HCEC monolayer. Measurable transendothelial migration of P5 was seen as early as 5 min after the addition to the luminal chamber of the BBB model (data not shown). Unrelated pentamerized sdAb did not show measurable migration across CEC monolayer (data not shown). Neither 10 nor 70 kDa dextran migrated across the same membranes (Fig. 3C).

4. Discussion

This study describes molecular engineering of the sdAb FC5 that selectively binds to and transmigrates across human CEC *in vitro* and the BBB *in vivo* to improve its binding and vector characteristics. FC5 was selected from the llama sdAb phage display library for its ability to transmigrate across the human *in vitro* BBB model and expressed and tagged for purification and detection purposes with His₅ and c-Myc [5,6], respectively. Both phage-displayed and soluble FC5 were then shown to cross the BBB *in vivo* and could be detected in the brain in contrast to unrelated sdAb [5]. Small size (13 kD) of sdAb, their resistance to proteases, and specific antigen recognition are advantageous for BBB delivery in comparison to either peptides or conventional antibodies [7]. However, sdAbs require further engineering for BBB ‘targeting’ or delivery applications. Firstly, llama sdAbs selected from naive phage display libraries exhibit relatively low binding activity in the low μM range [4,7]. Secondly, FC5 and FC44 have a limited number of available free groups (lysines, but not cysteine) for chemical attachment to therapeutic molecules. Thirdly, preliminary pharmacokinetics data in animals suggested that sdAbs have short half-life in plasma and are rapidly cleared by kidneys (unpublished observations).

To improve FC5 characteristics as a potential BBB carrier vector, a free cysteine was engineered at the C-terminus of FC5, 17 amino acids remote from the antigen-binding site. cysFC5 retained functional features of FC5, including transmigration across *in vitro* BBB model. Free cysteine group of cysFC5 was then used to chemically attach ‘model’ large molecule, HRP-IgG (~190 kDa) and it was demonstrated that only HRP-IgG ‘vectorized’ with FC5 entered human CEC and transmigrated across *in vitro* BBB, suggesting that sdAbs could successfully shuttle up to 10 times larger molecules into/across target tissues. Using similar chemical linking principles, large molecules of choice with potential therapeutic properties can be attached to cysFC5. Other chemical linker approaches that have been used for whole or single chain antibodies [13], including biotin–avidin linker, could also be employed with sdAbs providing that appropriate spacers are used to avoid steric hindrance with the antigen binding site. Given the ease with which sdAbs can be genetically engineered, alternative approaches to chemically linking therapeutic molecules are also possible, including chimeric (fusion) proteins. Similarly, bi-functional antibodies could be created by fusing two sdAbs with different antigen specificities.

The efficacy of recombinant antibodies is greatly improved by improving affinity by *in vitro* maturation [14] or multimerization. Multivalent single chain antibodies (scFv) have been previously generated by fusing to a helix-turn-helix peptide oligomerization domain

[15], the tetramerization domain of p53 [16], or by tetramerization on streptavidin [17]. In our initial studies on FC5 and FC44 transmigration across *in vitro* BBB model, we observed faster transport kinetics when sdAbs were expressed in the phage context (multimeric; 3–5 copies) than when they were in soluble (i.e., monomeric) form, despite the large size of phage particles. To obtain a proof of principle that multimeric display of BBB-targeting sdAb can potentially enhance BBB transport, we created pentameric FC5 construct with a VT1B homopentamer domain as recently reported for other sdAbs [9]. The reported model structure of sdAb pentabodies suggested a highly dynamic and flexible structure [9] that allows free interactions of each sdAb with the antigen, inducing antigen clustering effect. This pentabody formulation has been shown to increase binding to immobilized antigen(s) three to four orders of magnitude compared to monomeric version [9]. The P5 binding to LCM-captured brain vessels was 2-fold higher than that of monomeric FC5. Similarly, P5 uptake into HCEC and its transport across *in vitro* BBB models were faster when compared to monomeric FC5, indicating that antigen cross-linking is important in initiating endocytosis/transcytosis in CEC, similar to prior observations in other cells [18]. To exclude the involvement of VT1B subunit in binding and transmigration of P5, a mutant VT1B subunit that lacks the capability to bind Gb3 receptor [19] was used. A non-related pentameric antibody (ES1) failed to either bind to brain vessels or transmigrate across human CEC.

Although these studies provide evidence that multimeric presentation of BBB-targeting antibodies improves efficacy of vectorized delivery systems, the utility of P5 itself for this purpose is limited due to immunogenicity of VT1B [20]. However, P5 could be developed into an excellent reagent for immunohistochemical analyses of brain vessels since it selectively detected vessels in the brain of various species, but not in liver tissue sections. P5 will also find utility in identification of the CEC antigen involved in its transcytosis, since it demonstrated a strong binding to immobilized antigenic tissues (i.e., LCM-captured vessels). The advantage of pentameric sdAbs in identifying unknown antigens in tumor tissues has been recently demonstrated [21].

Engineering of BBB-permeable sdAbs, FC5 and FC44 to provide free linker moieties, such as that achieved with *cysFC5*, will enable alternative approaches for their multimeric display in the context of drug carriers. For example, *cysFC5* could be conjugated to polymeric components of nanoparticle delivery system [22] or to liposome-based particles using approaches similar to those reported for those reported for IgGs or scFvs [23]. These ‘containers’ vectorized with sdAbs could then be used to deliver drug payloads into the brain, a concept that has already been exploited using ‘classical’ antibodies against few known BBB antigens, including transferrin receptor [3,23].

The intact or injured BBB determines brain delivery of potential therapeutics, including neuroprotective agents. Designing formulations, carriers, particles, collectively designated ‘vectors’, to deliver macromolecules across the BBB remains a ‘holy grail’ of drug development for brain diseases. sdAb phage-display technology offers a unique flexibility for developing vectorized drug delivery systems since it allows for selection of antibodies against ‘disease’ phenotypes of targeted tissues, including specific phenotypes of brain vessels (e.g., angiogenic, inflammatory, etc). Relatively simple engineering of these ‘nanobodies’ will enable their future tailoring for applications in drug targeting, imaging or therapeutic approaches in both peripheral tissues and the brain.

Acknowledgements

Authors acknowledge the expert technical assistance of Ms. Ewa Bauman and Ms. Rita Ball.

References

- [1] D.J. Begley, M.W. Brightman, Structural and functional aspects of the blood–brain barrier, *Prog. Drug Res.* 61 (2003) 39–78.
- [2] I. Tamai, A. Tsuji, Transporter-mediated permeation of drugs across the blood–brain barrier, *J. Pharm. Sci.* 89 (11) (2000) 1371–1388.
- [3] U. Bickel, T. Yoshikawa, W.M. Pardridge, Delivery of peptides and proteins through the blood–brain barrier, *Adv. Drug Deliv. Rev.* 46 (2001) 247–279.
- [4] J. Tanha, et al., Selection by phage display of llama conventional V_H fragments with heavy chain antibody V_HH properties, *J. Immunol. Methods* 263 (1–2) (2002) 97–109.
- [5] A. Muruganadam, et al., Selection of phage-displayed llama single-domain antibodies that transmigrate across human blood–brain barrier endothelium, *FASEB J.* 16 (2002) 240–242.
- [6] J. Tanha, A. Muruganadam, D. Stanimirovic, Phage display technology for identifying specific antigens on brain endothelial cells, *Methods Mol. Med.* 89 (2003) 435–449.
- [7] S. Muyldermans, Single domain camel antibodies: current status, *J. Biotechnol.* 74 (4) (2001) 277–302.
- [8] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
- [9] J. Zhang, et al., Pentamerization of single-domain antibodies from phage libraries: a novel strategy for the rapid generation of high-avidity antibody reagents, *J. Mol. Biol.* 335 (1) (2004) 49–56.
- [10] D. Stanimirovic, et al., Angiotensin II-induced fluid phase endocytosis in human cerebromicrovascular endothelial cells is regulated by the inositol–phosphate signaling pathway, *J. Cell. Physiol.* 169 (3) (1996) 455–467.
- [11] J. Mojsilovic-Petrovic, et al., Development of rapid staining protocols for laser-capture microdissection of brain vessels from human and rat coupled to gene expression analyses, *J. Neurosci. Methods* 133 (2004) 39–48.
- [12] W. Zhang, et al., The expression and functional characterization of ABCG2 in brain endothelial cells and vessels, *FASEB J.* 7 (14) (2003) 2085–2087.
- [13] W.M. Pardridge, Vector-mediated drug delivery to the brain, *Adv. Drug Deliv. Rev.* 36 (2–3) (1999) 299–321.
- [14] R.A. Irving, et al., Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics, *J. Immunol. Methods* 248 (1–2) (2001) 31–45.
- [15] P. Pack, A. Plückthun, Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*, *Biochemistry* 31 (1992) 1579–1584.
- [16] J. Willuda, et al., Tumor targeting of mono-, di-, and tetravalent anti-p185(HER-2) miniantibodies multimerized by self-associating peptides, *J. Biol. Chem.* 276 (2001) 14385–14392.
- [17] S. Muro, et al., A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1, *J. Cell Sci.* 116 (2003) 1599–1609.
- [18] S.M. Cloutier, et al., Streptabody, a high avidity molecule made by tetramerization of in vivo biotinylated, phage display-selected scFv fragments on streptavidin, *Mol. Immunol.* 37 (17) (2000) 1067–1077.
- [19] A.M. Soltyk, et al., A mutational analysis of the globotriaosylceramide-binding sites of verotoxin VT1, *J. Biol. Chem.* 277 (7) (2002) 5351–5359.
- [20] D.J. Bast, et al., Toxicity and immunogenicity of a verotoxin 1 mutant with reduced globotriaosylceramide receptor binding in rabbits, *Infect. Immun.* 65 (1997) 2019–2028.
- [21] J. Zhang, et al., A pentavalent single domain antibody approach to tumor antibody discovery and the development of novel proteomics reagents, *J. Mol. Biol.* 341 (2004) 161–169.

- [22] A.V. Kabanov, E.V. Batrakova, New technologies for drug delivery across the blood brain barrier, *Curr. Pharm. Des.* 10 (12) (2004) 1355–1363.
- [23] J.C. Olivier, et al., Synthesis of pegylated immunonanoparticles, *Pharm. Res.* 19 (8) (2002) 1137–1143.

Discussion

Kreuter

I have just one question concerning absorptive endocytosis: Is that always energy-independent or are there cases where absorptive endocytosis can also be energy-dependent? That means it can be blocked at 4°.

Scherrmann

It can be energy-dependent. In some cases you need ATP.

De Boer

Since sialic acid residues are involved, is it feasible that some adhesion molecules are also involved in binding to these antibodies? Do you know something about that and about up- or down-regulation during disease states?

Stanimirovic

We haven't tested this particular single domain antibody in disease states. In the case of FC5 we see a very strong dependence on sialic acid residues for transcytosis; sialic acid abundance may change in disease states. We think that the antigen is sialiated protein. So, adhesion molecules are a possibility.

Given that the glycocalyx is so elaborate on brain endothelial cells, it could be glycosylated protein but also a ganglioside which is often sialiated, so we're not quite sure. We still don't have the identity of the antigen for FC5; what we know is that transport of the other antibody, FC44, is not sialic acid-dependent. This is really exciting because we now have two antibodies which obviously recognise different antigenic epitopes.

Smith

I was wondering if you knew if there was any possibility for regionally selective brain delivery based on protein targets that are only expressed in capillaries of certain brain regions. I would imagine that there are differences in brain capillary protein expression between white matter versus grey matter capillaries. This might be one mechanism for selective regional targeting.

Stanimirovic

Some time ago we looked into differences in endothelial cells that were cultured from capillaries, larger microvessels or large vessels and we found some differences. However it is a very difficult question to answer—some new technologies such as laser capture of vessels from specific brain areas or phage display may provide the answers in the future. There might be some regional differences in particular in disease conditions, because many neurodegenerative diseases are region-selective and affect brain vessels in a specific way.

Sugiyama

When you use this kind of approach or method the antigen should be internalised to some rate. When, several years ago, I looked at the literature I found that the antibody induced internalisation rate depends on the constitutive internalisation rate of the antigen. Therefore, I wonder if you could not find any method to detect the constitutive internalisation rate of the antigen. If you could do that, it would be very nice.

Stanimirovic

I completely agree with you. It is crucial to identify what the antigen is. Unfortunately we don't know that yet. What we know is that a sugar moiety is a component of the antigen. However, what I would like to point out is that endocytosis rates are much faster when we have a multimeric display of the antibody, which probably cross-links the antigen. We also noticed this when the antibody is expressed in the phage, where it is displayed in three to five copies. So it is possible that the constitutive rate of endocytosis is important, but I also think that endocytosis can be stimulated through cross-linking of the antigen.

Gaillard

I am always puzzled by the phage display technology and I think you did absolutely the right thing to start with human cells and human brain capillaries. Can you explain why it still works in mice or rats?

Stanimirovic

We were actually pleased that it worked in mice and rats. Many antigens expressed in the blood–brain barrier are common across species although some sugars may be different, as suggested by differential lectin binding. In this particular case, selection was done in the human system between peripheral endothelial cells and central endothelial cells, but we were actually happy to see that it works in mice and rats since it provides us with animal models for testing.

Van Tellingen

You've probably considered it already but I imagine that an obvious candidate for brain tumours would be VEGF receptor antibody—because it is not found in normal brain tissue and it's up-regulated in the brain tumour tissue—especially if you couple it to a directly cytotoxic agent. Are you already doing this kind of studies?

Stanimirovic

We considered the VEGF receptor but we decided against it. The fact that we did not select the VEGF receptor was mostly based on some of our own studies on glioblastoma sections where we could not see much expression in endothelial cells. But, I'm not discarding the receptor as a potential target. We are currently looking at other potential markers of angiogenesis or proliferating endothelial cells, such as endoglin, even though there is some dispute about whether this is the best marker or not. We are also considering fibronectin EDB fragment which gets exposed from the basement membrane when endothelial cells start proliferating. Our thought was to look at the imaging approach to show that we can see angiogenic vessels with some of these markers.

Abbott

I have a comment about these regional differences, which I think are going to be very important if one is trying to target specific parts of the nervous system. For example, there appear to be some differences in TNF alpha and leptin transport between brain and spinal cord. Recently, Sarah Thomas's group has shown that specific leptin transport may be higher, or at least more easily detectable, in cerebellum than in cortex and other parts of the brain (Kurrimbux et al. 2004, *Neurosci.* 123:527–36). So there may already be some tissues that you could use as your selection for comparison.