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Drug discovery and the BBB

Exploiting transferrin receptor for delivering drugs across the blood-brain barrier

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Delivery of large molecule drugs across the blood brain barrier is increasingly being seen as an achievable goal. Several technologies have been described where following peripheral administration the molecules can be detected in the brain. Foremost amongst these technologies are antibodies against the transferrin receptor. Following a burst of publications in the very early twenty first century, excitement seemed to wane as contrary data started to emerge. Over the last few years antibodies against transferrin receptor have again started to raise hopes of successful drug delivery to the central nervous system, as protein engineering techniques have allowed a more detailed understanding of the antibody properties necessary for successful transport across the blood brain barrier.

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Introduction

The development of antibody-based therapeutics has taken huge steps forward in recent decades in all but a few fields. The brain has remained inaccessible to immunotherapies as a result of the barrier that separates it from the systemic circulation; the blood:brain barrier (BBB). Specialised tight junctions between the endothelial cells that make up the front line of the BBB mean that whilst a small amount of most antibodies will gain access to the brain after peripheral injection, this is reported to be as low as 0.1% of injected dose [1]. Therefore most antibodies would have to be administered at very high levels in order to produce a therapeutic response, which could lead to potentially toxic side effects. In this review we will focus on one receptor that has been targeted, the transferrin receptor, and discuss the challenges faced in transporting large molecule therapeutics across the BBB.

Transferrin receptor as a route into the brain

Iron is an important molecule for brain function but must be imported across the BBB for use by the cells of the brain, and transferrin is the primary iron transporting protein [2]. Iron-loaded transferrin (holo-transferrin) binds to transferrin receptors on the surface of microvascular endothelial cells and is transported across the BBB to the brain [3]. There is some uncertainty as to whether iron is dissociated from transferrin within the low pH endosomal compartments of the brain endothelial cells before release on the abluminal face or whether the holo-transferrin complex dissociates from the transferrin receptor within endosomes, however it is clear that the transferrin receptor must transcytose from the blood to the brain side of the barrier to release its cargo. Technologies exploiting this transport pathway have been developed that have the potential to deliver much needed treatments to the central nervous system.

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In response to the challenges that the BBB presents, novel methods of delivering therapeutics to the brain are required. A main focal point for brain targeting technology development over the last couple of decades has been the so-called 'Trojan horse' method [4]. In this method an antibody is developed that binds to an endogenous receptor on the luminal face of the BBB endothelial cells. As the receptor is internalised and transported through the cells, the antibody is transported alongside and then released on the abluminal side. Once the antibody has been established as a BBB-crossing technology, it can be used to shuttle therapeutic molecules into the brain to treat otherwise unreachable conditions, for example, brain tumours, Alzheimer's disease and Parkinson's disease.

Antibodies against transferrin receptor: unfulfilled promise

The ability of transferrin receptor to bind and promote the cellular uptake of a large protein lends itself to exploitation for the development of an engineered solution for the delivery of recombinant proteins, such as antibodies, across the BBB. Two antibodies in particular have been the subject of extensive study: OX26 is a mouse monoclonal antibody which recognises rat transferrin receptor [5] and 8D3 is a rat antibody raised against the mouse transferrin receptor [6]. Both antibodies have been shown to bind to transferrin receptor and internalise into brain endothelial cells without interfering with the uptake of iron-bound transferrin. Jefferies *et al.* [5] characterised the OX26 antibody as binding to branched capillary structures in cryosections of the rat brain, but not to the vasculature of other organs. They also showed that intravenous injection of the OX26 antibody resulted in a staining pattern that was indistinguishable from that of a Factor VIII antibody, an established capillary marker. In addition, a publication from Friden *et al.* [7] showed that injection of OX26 resulted in detection of the antibody in the brain vasculature of rats after one hour. A maximum value of 0.44% of the injected dose of OX26 was detectable in the capillary-depleted fraction of the brain after 24 hours, suggesting that the antibody was able to cross the BBB and access the parenchyma. A subsequent publication by Pardridge *et al.* [8] demonstrated that OX26 was able to bind to isolated capillaries from bovine, human and rat brain, suggesting that it bound a conserved transferrin receptor epitope. In addition, they repeated the capillary depletion experiments and showed again that OX26 was detectable in the fraction representing the brain parenchyma.

A study into the transport of OX26 across the BBB in rats showed that the kinetics of OX26 were very different from those of a non-immune isotype control antibody, with OX26 being cleared from plasma much more quickly [9]. However, the authors observed that OX26 accumulated within the brain at a much higher level than the control IgG following i.v. injection. Subsequent experiments using a capillary-depletion technique demonstrated that, in contrast with previous studies, 90–95% of the amount of detectable OX26 was associated with the capillary-containing pellet. This was confirmed with immunohistochemistry data showing that OX26 was mainly associated with brain capillaries throughout the parenchyma. Interestingly, they also saw that OX26 was associated with neurons only in areas that were in close proximity to the ventricular system, suggesting that OX26 may have been accessing the brain via the blood-CSF barrier or cerebroventricular organs. Detection of OX26 in the CSF appeared to support this theory. The authors discussed the problems of a high-affinity antibody-antigen interaction that would be difficult to reverse in order for OX26 to be released from the abluminal surface of brain endothelial cells following transcytosis via the transferrin receptor, evidence for which would come over a decade later.

An initial study into the targeting of 8D3 showed that it could be detected in the brain one hour after injection [10]. However, contrary to reports in articles that cite this original study, whilst experimental capillary depletion data was provided for one of the anti-transferrin receptor antibodies, Ri7, no data was presented for 8D3. Therefore, it is impossible to conclude from this study whether the 8D3 antibody had reached the brain parenchyma, or whether it was retained in the capillaries. Despite this lack of evidence for brain penetration a number of studies were performed with 8D3 coupled to a variety of therapeutic or biologically active molecules, such as targeted pegylated immunoliposomes [11,12], radiolabelled peptides [13], antisense oligos [14], and an enzyme [15]. All of these studies have concluded that 8D3 is able to penetrate into the brain parenchyma, but they often focus on a single time point and/or lack appropriate controls. For example; studies targeting 8D3 fusions to an antisense oligo [14], to beta-galactoside [15] and to an A-beta peptide [13] compare the 8D3-conjugated cargo to the free cargo. The difference in the pharmacokinetics between the cargo when conjugated to an antibody and the free cargo makes it difficult to conclude whether or not it is 8D3 that is conferring brain exposure. An irrelevant antibody fused to the cargo would have been a far more informative control.

More recent work investigating the fate of anti-mouse transferrin receptor antibodies injected systemically showed that the 8D3 antibody bound to the endothelial cells of the brain vasculature but found little evidence to suggest that the antibodies were able to make their way deeper into the brain parenchyma [16]. However, with the use of confocal microscopy, they were able to see some co-localisation with collagen IV, which is a commonly used marker for the basal lamina, suggesting that the antibodies were able to cross from the luminal to the abluminal side of the brain endothelial cells, but were not released from the abluminal transferrin receptor.

Recent data and realisation of potential

To be useful as a targeting system an anti-transferrin receptor antibody needs to be able to deliver its cargo to the brain parenchyma over an extended time frame consistent with the peripheral half-life of the antibody, and show a significant improvement over an isotype control antibody fused to the same cargo. In 2011, a paper from researchers at Genentech led to resurgence in interest in the transferrin receptor as a 'Trojan horse' for drug delivery across the brain. Yu *et al.* [17] proposed that using an antibody with decreased affinity for the transferrin receptor would allow release of the antibody on the abluminal side of the BBB. Their hypothesis stated that using a therapeutic dose of the lower affinity antibody rather than a trace dose would overcome any issues that arose as a result of the decreased level of binding. A panel of anti-mouse transferrin receptor antibodies with a range of different affinities was generated against the mouse transferrin receptor and it was demonstrated that although the highest affinity antibody showed higher accumulation in the brain compared with a control IgG following a therapeutic dose, the majority was located in the capillaries of the brain even after 24 hours when the control antibody could no longer be detected. Investigating the panel of lower affinity mutant antibodies dosed i.v. at a therapeutic level showed that the level of antibody detectable in the brain after 24 hours was inversely correlated with the antibody affinity for the transferrin receptor. Immunohistochemical analysis of perfused brains 24 hours after injecting the lower affinity antibodies showed parenchymal localisation and some co-localisation with a neuronal marker.

Further work from the same group offered some evidence for a potential mechanism for the lower levels of brain exposure of the high affinity transferrin receptor antibody. Bien-Ly *et al.* [18] published details of an increase in the internalisation and subsequent lysosomal degradation of transferrin receptor following incubation with a high affinity anti-transferrin receptor antibody compared to a lower affinity or control antibody. The authors proposed that this increased degradation of transferrin receptor contributed to the lower brain exposure of high affinity anti-transferrin receptor antibody as the antibody was degraded along with the receptor, and the recycling of transferrin receptor that would usually occur to bind more antibody at the cell surface could not take place.

A second group also published data on the importance of avoiding lysosomal degradation when targeting the brain via the transferrin receptor, although they proposed that avidity rather than affinity was the important factor in determining the fate of an anti-transferrin receptor antibody at the BBB. Niewoehner *et al.* [19] hypothesised that monovalent rather than divalent binding of an antibody would better mimic the monovalent binding of the endogenous ligand and lead to better penetration into the brain. A direct comparison of monovalent (mFab) *vs* divalent (dFab) forms of an anti-mouse transferrin receptor antibody showed *in vitro* that although dFab was internalised at a faster rate, significantly more of it was associated with a lysosomal marker than mFab. They also showed that there was a reduced amount of transferrin receptor recycled to the cell surface in response to treatment with dFab, whereas mFab had no effect on the normal transferrin receptor pathway. The two formats of the mouse antibody were also used *in vivo* conjugated to an anti-amyloid plaque antibody (mAb31) to look for target engagement on the brain side of the BBB. mFab appeared to cross the endothelial cells of the BBB and distribute throughout the parenchyma where the mAb31 was able to engage with and decorate amyloid plaques, whereas dFab was restricted to the vasculature and again associated with a lysosomal marker. The authors discussed the possibility that divalent binding of the antibody to the transferrin receptor may be causing receptor dimerization and promoting trafficking to the lysosome. In addition to affinity and avidity, there is evidence from another publication that pH-dependent receptor binding may be important in determining the sorting pathway of an anti-transferrin receptor antibody [20]. Comparing various commercially available antibodies it was demonstrated that those that exhibited less efficient binding to transferrin receptor in the lower pH environment of the late endosome could be detected in the lower well of an *in vitro* transcytosis experiment whereas pH-independent binders remained trapped within cells.

Our own data has indicated that both 8D3 and OX26 are able to efficiently bind to brain endothelial cells, but do not penetrate further into the brain. Engineering either to reduce their affinity for transferrin receptor results in a significant increase in accumulation within the brain parenchyma (unpublished) and supports the reduced affinity hypothesis.

In reality it is likely that all of the parameters described above, affinity, avidity and pH dependence, are likely to play a part in the trafficking of anti-transferrin receptor antibodies across the BBB. Only through the systematic evaluation of all of these parameters will it become clear which, if any, is the most important.

Path to the clinic

Alongside the positive aspects of targeting the transferrin receptor for brain delivery of therapeutics, a note of caution must also be raised. Transferrin receptor is expressed on cells other than those of the brain endothelium and a study that investigated the safety aspects of dosing with anti-transferrin receptor antibodies found that a single dose of a high affinity anti-transferrin receptor antibody caused acute clinical signs and reticulocyte depletion in mice, even at low doses [21]. Reducing the affinity of the antibody attenuated the reticulocyte destruction in a dose-dependent manner. However, it appeared to be effector function that was responsible for the

appearance of clinical signs and abolishing effector function by introducing mutations into the Fc domain of the monospecific antibody eliminated the clinical signs although some reticulocyte depletion was still observed.

Perhaps the most challenging aspect of moving anti-transferrin receptor antibodies to the clinic is the lack of species cross reactivity observed in the available antibodies. Transferrin receptor is not highly conserved between species at the level of the amino acid sequence and obtaining an antibody that can be used for both pre-clinical studies and clinical products may prove to be very difficult, especially as the affinity towards each species of transferrin receptor is likely to need to be maintained. The solution is to isolate an antibody for use in each species under investigation, or perhaps one for use in rodents, and one for primates, something that will add significantly to development costs.

Conclusions

For now, anti-transferrin receptor antibodies may not be the answer to the biologics brain targeting question. However the research performed with the available antibodies has provided invaluable insight into the mechanisms of action of receptors at the BBB and has also helped to highlight protein engineering issues that must be addressed in order for a successful BBB shuttle to be developed.

Conflict of interest

JP and CIW are employees of MedImmune, a company engaged in research into the transport of antibodies across the blood brain barrier, and therefore have a theoretical conflict of interest with regard the publication of this review.

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