The Transferrin Receptor at the Blood-Brain Barrier - exploring the possibilities for brain drug delivery

Visser, Corine

The transferrin receptor at the blood-brain barrier: Exploring the possibilities for brain drug delivery

The transferrin receptor at the blood-brain barrier: Exploring the possibilities for brain drug delivery

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D.D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 18 januari 2005
klokke 14.15 uur

door

Cornelia Christina Visser

geboren te Gorinchem

in 1977

Promotiecommissie

Promotores:	Prof. Dr. M. Danhof
	Prof. Dr. D.J.A. Crommelin, UIPS, Universiteit Utrecht
Co-promotor:	Dr. A.G. de Boer
Referent:	Dr. T. Moos, The Panum Institute, Kopenhagen, Denemarken
Overige leden:	Prof. Dr. Th.J.C. van Berkel
	Prof. Dr. E.R. de Kloet
	Prof. Dr. G.J. Mulder
	Prof. Dr. A.P. IJzerman

Daarom lijkt het me het beste dat de mens vrolijk is en geniet van het leven.
Want als hij eet en drinkt en plezier heeft van zijn werk, is dat een geschenk van God.

Prediker 3: 12,13 (Groot Nieuws Bijbel)

Aan mijn ouders

The research described in this thesis was performed at the Division of Pharmacology of the Leiden/Amsterdam Centre for Drug Research (LACDR). This research was part of the NDRF project "Targeting of CRH-receptor antisense probes to and in the central nervous system. Application in neuropsychiatry", a collaboration between the universities of Groningen and Leiden and Solvay Pharmaceuticals. This research was supported by STIGO (stimulation for innovative drug research, ZonMW) grant 014-80-003, the Hague, the Netherlands.

The printing of this thesis was financially supported by:

LACDR
Solvay Pharmaceuticals
to-BBB technologies BV

Printed by Ponsen & Looijen, Wageningen, the Netherlands

Cover design: Corine Visser, created by Ponsen & Looijen
The pictures on the cover are made at "de Achelse Kluis", a monastery located at the Dutch-Belgium border.

ISBN: 90 6464 7208

Contents

Section 1

General Introduction

Chapter 1

Preface:

Scope and intent of the investigation

Background of the investigations

There are many diseases of the central nervous system (CNS), like Parkinson's disease, Alzheimer's disease, depression, schizophrenia, epilepsy, migraine headache, and HIV infection in the brain (1). However, treatment is difficult since many drugs cannot reach the brain in sufficient quantities due to the existence of the blood-brain barrier (BBB). According to Pardridge (2001), more than 98 % of all potential new drugs for the treatment of CNS disorders do not cross the BBB (1). Over the last years, pharmaceutical companies have focussed on the development of small drug molecules as therapeutic moieties. In general, small molecules should be lipid-soluble and have a molecular weight below 400 – 600 Da to be able to cross the BBB in therapeutically effective quantities (1). These characteristics often cannot be found in one molecule and, therefore, many of these small drug molecules will not cross the BBB in sufficient quantities without brain drug-targeting strategies. In addition, more and more larger molecules are generated by biotechnological means which constitute promising alternatives for the treatment of diseases of the CNS. These include proteins (neurotrophins, (2)) or genes (neprylysin gene, (3)) for Alzheimer's Disease, antisense therapy for Huntington's disease (4) and monoclonal antibodies for diagnostic purposes (5) and the treatment of brain metastasis of breast cancer (6, 7). These larger biotechnology therapeutics can not cross the BBB without using targeting and delivery strategies. Promising targeting strategies for drug delivery to the brain often focus on endogenous transporters at the BBB, such as the insulin receptor (8), the transferrin receptor (9, 10) or the hexose transport system for glucose and mannose (11). Targeting and delivery strategies include the use of pro-drugs, recombinant proteins, drug-targeting vector conjugates or liposomes tagged with a targeting vector.

Scope and outline of this thesis

The aim of this thesis is to explore the possibilities for drug targeting to the brain by using the transferrin receptor (TfR) at the BBB. These studies are conducted *in vitro*, with primary cultured bovine brain capillary endothelial cells (BCEC). Gaillard *et al* (2001) have developed an *in vitro* model of the BBB where BCEC are co-cultured with rat

astrocytes (12). However, we have not co-cultured the BCEC in the presence of astrocytes, but we used astrocyte-conditioned medium to be able to investigate the TfR more mechanistically. Bovine transferrin (Tf) is used as a targeting vector since the available bovine polyclonal antibodies are not specific enough for drug targeting purposes. Furthermore, by using the endogenous ligand a more mechanistic approach to explore the possibilities for drug targeting to the TfR was possible.

Chapter 2 gives an introduction to the biology and physiology of the BBB, with emphasis on the TfR. Furthermore, drug targeting and delivery strategies to the brain are discussed. *Section 2* of this thesis focuses on the TfR. **Chapter 3** describes the characterisation of the TfR on BCEC *in vitro*. In addition, the influence of several modulators, such as an excess of iron, an iron scavenger, astrocyte conditioned medium and lipopolysaccharide (LPS) on the expression and internalisation of the TfR is investigated. In **chapter 4** the validation of the TfR for drug targeting is described. For this research conjugates of horseradish peroxidase (HRP) and Tf were prepared. HRP is a 40 kDa protein, which normally does not cross the BBB. It was found that Tf-HRP conjugates were internalised by the TfR via a similar mechanism as endogenous Tf.

Section 3 focuses on Tf-tagged liposomes. By using liposomal drug carriers, the ratio of drug molecules per targeting vector (i.e. Tf) is increased for certain classes of drug molecules. Furthermore, it is not necessary to chemically modify the drug, as is the case for drug conjugates. The preparation of Tf-tagged liposomes is described in **chapter 5**. For this preparation it is essential that Tf retains its two iron atoms, since di-ferric Tf has a much higher affinity for the TfR than apo-Tf. Subsequently, in **chapter 6** the Tf-tagged liposomes are loaded with HRP to determine the association of liposomes by BCEC in vitro. This research showed that Tf-tagged liposomes were suitable for drug targeting to the brain. However, Tf-tagged liposomes had a different intracellular distribution than Tf-drug conjugates.

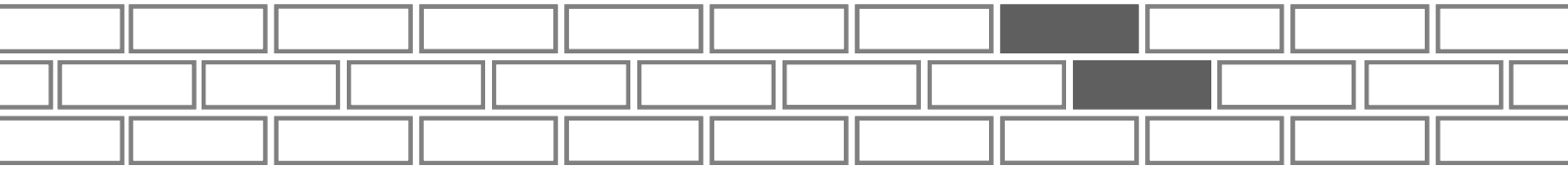
In *section 4* drug targeting and delivery under inflammatory disease conditions is described. For this research, Tf-tagged liposomes containing the free radical scavenger N-acetyl-L-cysteine were prepared. However, we found that liposomes themselves interacted with LPS. In **chapter 7** this interaction of liposomes and LPS is investigated and the influence of time of incubation, presence of serum and liposome composition are described.

To conclude this thesis, **chapter 8** summarises and discusses the results that were obtained and some future perspectives are presented.

References

1. Pardridge, W. M. (2001) Drug targeting, drug discovery, and brain drug development. In *Brain drug targeting - The future of brain drug development* (W. M. Pardridge, ed.), University Press, Cambridge, pp. 1-12
2. Pardridge, W. M. (2002) Neurotrophins, neuroprotection and the blood-brain barrier. *Curr Opin Investig Drugs* **3** (12): 1753-1757
3. Mohajeri, M. H., Kuehnle, K., Li, H., Poirier, R., Tracy, J. and Nitsch, R. M. (2004) Anti-amyloid activity of neprilysin in plaque-bearing mouse models of Alzheimer's disease. *FEBS Lett* **562** (1-3): 16-21
4. Gutekunst, C. A., Levey, A. I., Heilman, C. J., Whaley, W. L., Yi, H., Nash, N. R., Rees, H. D., Madden, J. J. and Hersch, S. M. (1995) Identification and localization of huntington in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. *Proc Natl Acad Sci U S A* **92** (19): 8710-8714
5. Wu, D., Yang, J. and Pardridge, W. M. (1997) Drug targeting of a peptide radiopharmaceutical through the primate blood-brain barrier in vivo with a monoclonal antibody to the human insulin receptor. *J Clin Invest* **100** (7): 1804-1812
6. Grossi, P. M., Ochiai, H., Archer, G. E., McLendon, R. E., Zalutsky, M. R., Friedman, A. H., Friedman, H. S., Bigner, D. D. and Sampson, J. H. (2003) Efficacy of intracerebral microinfusion of trastuzumab in an athymic rat model of intracerebral metastatic breast cancer. *Clin Cancer Res* **9** (15): 5514-5520
7. Bendell, J. C., Domchek, S. M., Burstein, H. J., Harris, L., Younger, J., Kuter, I., Bunnell, C., Rue, M., Gelman, R. and Winer, E. (2003) Central nervous system metastases in women who receive trastuzumab-based therapy for metastatic breast carcinoma. *Cancer* **97** (12): 2972-2977
8. Duffy, K. R. and Pardridge, W. M. (1987) Blood-brain barrier transcytosis of insulin in developing rabbits. *Brain Res* **420** (1): 32-38
9. Huwyler, J. and Pardridge, W. M. (1998) Examination of blood-brain barrier transferrin receptor by confocal fluorescent microscopy of unfixed isolated rat brain capillaries. *J Neurochem* **70** (2): 883-886
10. Qian, Z. M., Li, H., Sun, H. and Ho, K. (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharmacol Rev* **54** (4): 561-587
11. Tsuji, A. and Tamai, I. I. (1999) Carrier-mediated or specialized transport of drugs across the blood-brain barrier. *Adv Drug Deliv Rev* **36** (2-3): 277-290
12. Gaillard, P. J., Voorwinden, L. H., Nielsen, J. L., Ivanov, A., Atsumi, R., Engman, H., Ringbom, C., de Boer, A. G. and Breimer, D. D. (2001) Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur J Pharm Sci* **12** (3): 215-222

Chapter 2



Drug delivery to the brain:

The transferrin receptor as target

Parts of this chapter will be published in an invited review

in Expert Opinion on Drug Delivery

Contents Chapter 2

1. Introduction

The central nervous system is protected by the blood-brain barrier. This barrier limits the transport of exogenous compounds and controls the selective and specific transport of nutrients to the brain. Unfortunately, drugs for the treatment of brain diseases are often not able to cross the blood-brain barrier. Therefore, various drug targeting and delivery strategies are being developed.

This chapter discusses the biology and physiology of the BBB, with a focus on endogenous transport mechanisms, in particularly, the transferrin receptor. Finally, a selection of drug targeting and delivery strategies is reviewed and discussed.

2. The Blood-Brain Barrier

The blood-brain barrier (BBB) is situated at the interface of blood and brain and its primary function is to maintain the homeostasis of the brain. In addition to the BBB, there is a second barrier at the blood - cerebrospinal fluid (CSF) interface, presented by the choroid plexus epithelium (1). Furthermore, the BBB is not uniform throughout the brain, since the capillaries in the circumventricular organs (CVO's) are fenestrated (2, 3). Figure 1 gives a schematic representation of the barriers present in the CNS.

The human BBB has a total blood vessel length of approximately 650 km, and an estimated surface area of approximately 20 m^2, which makes it about 1000 times larger than the blood-CSF or the brain-CSF barrier (4). Therefore, the BBB is considered the most important barrier for solutes to reach the brain (2, 3).

The first evidence for the existence of a barrier between blood and brain was discovered by Ehrlich (1885), who injected the dye trypan blue intravenously and found that, in contrast to other tissues, it did not stain the brain (5). In a second experiment Goldman (1913) injected the dye into the cerebral spinal fluid, after which staining of the brain was observed, but not of the peripheral organs (6). After this discovery, much research has been performed on the biology and physiology of the BBB.

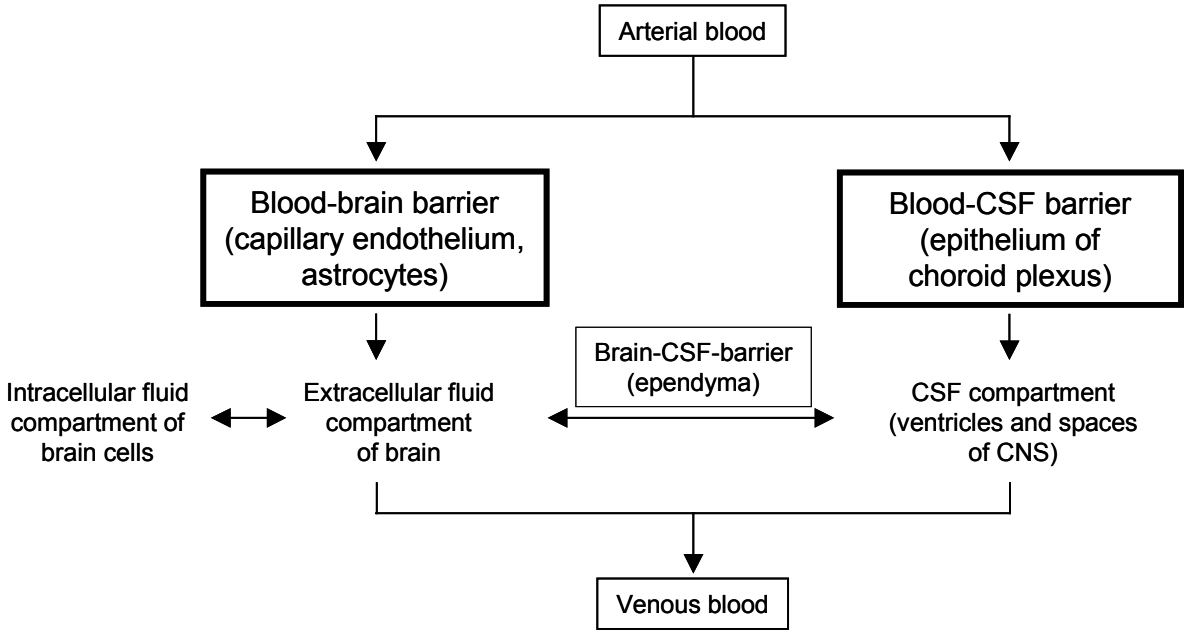


Figure 1: Schematic representation of the blood-brain barrier (BBB), the blood-cerebrospinal fluid (CSF)-barrier and the brain-CSF-barrier. The BBB has the largest surface area, and is therefore considered to be the most important barrier for solutes to reach the brain.

2.1 Blood-brain barrier biology

The BBB is mainly formed by brain capillary endothelial cells (BCEC) (7), although other cells such as, astrocytes, pericytes and neuronal cells also play an important role in the function of the BBB (8). BCEC are different from peripheral endothelial cells, as can be seen schematically in figure 2. BCEC have specific characteristics, such as tight junctions, which prevent paracellular transport of small and large (water soluble) compounds from blood to the brain (7, 9, 10). Furthermore, transcellular transport from blood to brain is limited as a result of low vesicular transport, high metabolic activity and a lack of fenestrae (8). These specific characteristics of the BBB are induced and maintained by the (endfeet of) astrocytes, surrounding the BCEC (7, 11), as well as by neuronal endings, which can directly innervate the BCEC (8, 12). Pericytes also play a role at the BBB, as they share the capillary basement membrane with the BCEC. Their phagocytotic activity forms an additional BBB property (2). Because of these complex interactions between cell types, as well as the dynamic regulation of the BBB properties

(e.g. receptor expression, formation of tight junctions) the BBB is considered to be an organ protecting the brain (13).

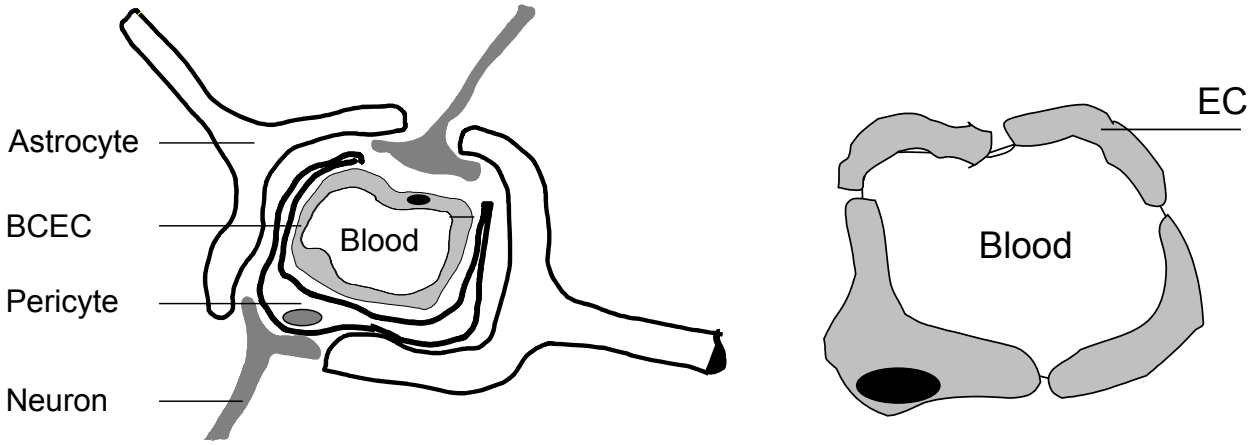


Figure 2: Comparison between a brain capillary endothelial cell (BCEC, left) and a peripheral endothelial cell (EC, right). See text for details. This picture is adapted from Pardridge (8)

2.2. Blood-brain barrier physiology

The function of the BBB is to exclude toxic exogenous compounds from the brain, and to nourish the brain with essential nutrients, such as ions, glucose, amino acids, purines, nucleosides, peptides and proteins (14, 15). Several influx mechanisms exist at the BBB, which can be divided into active or passive BBB transport mechanisms (figure 3). Passive diffusion depends on lipophilicity and molecular weight (16).

Furthermore, the ability of a compound to form hydrogen bonds will limit its diffusion through the BBB (17). In general, Lipinski's rule-of-5, as well as the Abraham's equation can be used to predict the passive transport of a drug molecule across the BBB (18, 19). Transport of hydrophilic compounds via the paracellular route is limited, while lipophilic drugs smaller than 400 – 600 Da can enter the brain via the transcellular route. Active transport systems can be divided into carrier-mediated (CMT), absorptive-mediated (AMT), or receptor-mediated transcytosis (RMT).

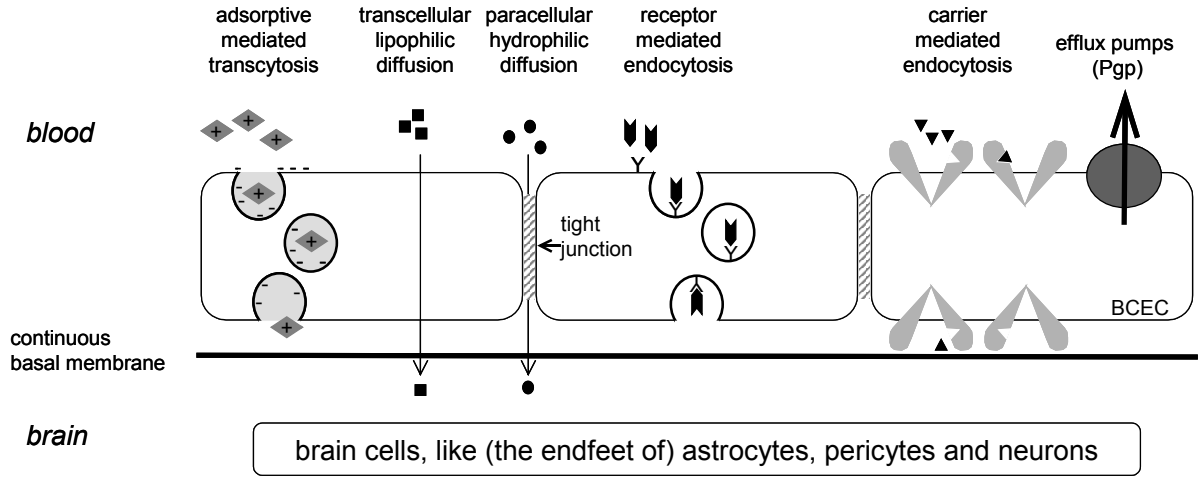


Figure 3: Schematic representation of the transport mechanisms present at the BBB. See text for more details about the transport mechanisms. This picture is adapted from Abbott and Romero (20).

CMT is used for the transcytosis of nutrients, such as glucose, amino acids and purine bases (20, 21). At least eight different nutrient transport systems have been identified, which each transport a group of nutrients of the same structure. Examples are the hexose transporter, which transports glucose and mannose, and the amino acid transporters, which can be roughly subdivided into anionic-, cationic- or neutral amino acid carriers (22). CMT is selective and the transport rate is dependent on the occupation rate of the carrier (21).

AMT is initiated by the binding of polycationic substances to negative charges on the plasma membrane (23, 24). This process does not involve specific plasma membrane receptors. Upon binding of the cationic compound to the plasma membrane, endocytosis occurs, followed by the formation of endosomes.

Peptides and proteins can undergo transport to the brain via RMT. Examples of receptors involved in RMT are the insulin receptor (25), the transferrin receptor (26, 27), and the transporters for low-density lipoprotein (28), leptin (29) and insulin-like growth factors (30). In general, RMT occurs in 3 steps: receptor-mediated endocytosis of the compound at the luminal (blood) side, movement through the endothelial cytoplasm, and exocytosis at the abluminal (brain) side of the brain capillary endothelium (2).

Besides many influx mechanisms, several efflux mechanisms exist at the BBB. The best known is P-glycoprotein (Pgp). Pgp is a transmembrane protein, located at the

apical membrane of the BCEC. It has a high affinity for a wide range of compounds, including cytotoxic anticancer drugs, antibiotics, hormones and HIV protease inhibitors (31). Other multidrug resistance (MDR) efflux mechanisms at the BBB include the MDR related protein (MRP), such as MRP 1, 2, 5 and 6 (32).

In addition, many other transporters are present at the BBB, like the organic anion transporter (influx and efflux), the organic cation transport system (influx) and the nucleoside transporter system (influx) (13, 33).

In conclusion, research over the years has shown that the BBB is a dynamic system, which combines restricted diffusion to the brain for exogenous compounds with specialised transport mechanisms for essential nutrients.

3. The transferrin receptor

The transferrin receptor (TfR) is a transmembrane glycoprotein consisting of two 90 kDa subunits (figure 4). A disulfide bridge links these subunits and each subunit can bind one transferrin (Tf) molecule (27). The TfR is expressed mainly on hepatocytes, erythrocytes, intestinal cells, monocytes, as well as on endothelial cells of the BBB (34, 35). Furthermore, in the brain the TfR is expressed on choroid plexus epithelial cells and neurons (27). The TfR mediates cellular uptake of iron bound to transferrin (Tf).

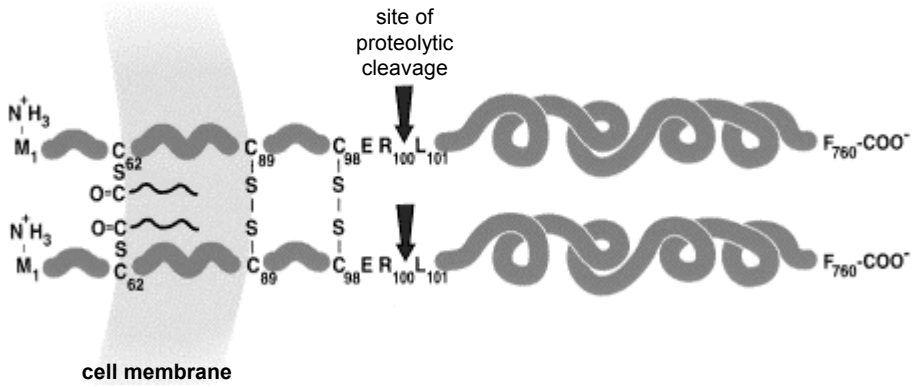


Figure 4: Schematic representation of the transferrin receptor, which is a transmembrane, homo-dimer glycoprotein. Arrows indicate the site of proteolytic cleavage. Standard one-letter abbreviations for amino acids are used (C, cysteine; E, glutamate; L, leucine; M, methionine; F, phenylalanine; R, arginine). Source: http://www.rndsystems.com/asp/g_sitebuilder.asp?bodyId=226

The expression level of the TfR depends on the level of iron supply and rate of cell proliferation. For example, in malignant cells an elevated level of TfR expression is found. This is caused by the high iron requirements for malignant growth (35, 36). The iron concentration determines TfR synthesis and expression via an iron-responsive element (IRE) in the mRNA of the TfR (37, 38). This IRE is also found in the mRNA of ferritin, a protein that can store iron (37). In cases of low iron concentrations, a so-called IRE binding protein stabilises the mRNA of the TfR, which can therefore be translated. The mRNA of ferritin is in low-iron situations less stable and is therefore translated to a lesser extent.

Recently, a second TfR (TfR-2) has been identified (39), which does not contain an IRE in its mRNA. TfR-2 is differentially distributed from TfR and has a 25-fold lower affinity for Tf. Finally, a soluble or serum TfR is present in the circulation (40). During the process of recycling of the TfR, some receptors are shed, in which case they appear in truncated form in the blood circulation (41). It has been shown that serum TfR to ferritin ratios have significant predictive value for differentiating iron deficiency anaemia from non-iron deficiency anaemia (42).

3.1. Transferrin

Tf, the natural occurring ligand for the TfR, is a member of the family of Fe-binding glycoproteins, which also includes lactoferrin, melanotransferrin and ovotransferrin. (34). Plasma Tf is mainly synthesised in the liver, but similar proteins are also synthesised in the brain, testes, and mammary glands. In the brain, Tf mRNA has been found in choroid plexus epithelial cells, oligodendrocytes, astrocytes, and neurons. However, the oligodendrocytes appear to be the major source of brain-derived Tf (27, 34). Furthermore, Tf in the brain is found in neurons and BCEC, although this Tf is probably derived from the extracellular fluid, blood plasma, and brain interstitial fluid by receptor-mediated endocytosis (27).

Tf is a single chain, 80 kDa protein, which is folded into two lobes (27) (figure 5). Each lobe of the Tf molecule can bind one iron ion, a binding that is virtually irreversible at physiological pH (27). Iron is being released as the pH is lowered to values below 6.5. In plasma and other extracellular fluids, Tf is present as a mixture of iron free

(apo-Tf), monoferric Tf, and diferric Tf (holo-Tf). The relative abundance of each form depends on the concentrations of iron and Tf (27, 43).

Tf can also bind other metals, such as aluminium, cadmium, manganese or copper, albeit with lower affinity. It has not been determined yet whether the binding of these metals has physiological significance (27).

Figure 5: A model of human serum Tf, loaded with 2 iron atoms, one in each lobe.
Source: http://srs.dl.ac.uk/arch/DALAI/biology_II.html

3.2. Transferrin receptor – transferrin interaction

Upon binding of Tf to its receptor, the receptor-ligand complex is endocytosed via clathrin-coated vesicles (figure 6, (44)). Subsequently, the endosomes that are formed are acidified to approximately pH 5.5. At low pH Fe^{3+} is released from Tf, and transported to the cytosol via the divalent metal transporter 1 (DMT-1) (44, 45). The remaining apo-Tf has a high affinity for the TfR at low pH and is recycled back to the luminal side of the BCEC. At physiological pH apo-Tf is released from the TfR and is able to acquire iron again. The intracellular Fe^{3+} can be stored in ferritin, or it can be used for mitochondrial activity (44). Furthermore, Fe^{3+} can be exocytosed at the abluminal side,

probably via ferroportin-1, hephaestin and/or hephaestin independent export systems (46).

There is also a second mechanism proposed in which diferric Tf crosses the BBB. Huwyler and Pardridge (1998) have shown that the TfR is present on the abluminal membrane of BCEC (47). Further research by Zhang and Pardridge (2001) has revealed that Tf is rapidly effluxed from the brain and that the efflux of apo-Tf exceeds that of holo-Tf (48). These results indicate that there is a bi-directional transport of Tf (45, 48). However, it has been shown that the iron transport across the BBB exceeds the Tf transport (27). Therefore, the mechanism in which Tf returns to the luminal side after releasing Fe^{3+} intracellularly, is considered the most likely.

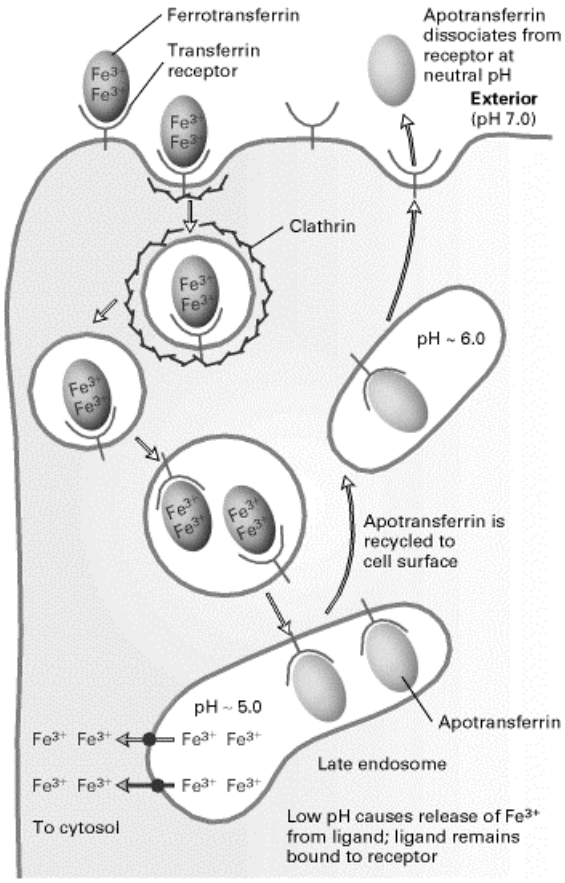


Figure 6: Schematic representation of the TfR internalisation upon binding of Tf to its receptor. See text for details. Source: Molecular Cell biology (section 17.9)

4. Drug targeting and delivery strategies to the brain

For many diseases of the brain, such as Alzheimer's disease, Parkinson's disease, depression, schizophrenia, epilepsy, migraine headache, and HIV infection in the brain no effective drugs are on the market (4). Part of the problem may be the poor BBB penetration of most of the newly developed drugs for treatment of these disorders. This includes approximately 98% of the small molecules and nearly 100% of large molecules, such as recombinant proteins or gene-based medicines (49). Therefore, much effort is put towards targeting and delivery of drugs to the brain. Drug delivery to the brain can be achieved via several methods, including invasive, pharmaco-chemical or physiological strategies.

4.1. Small drug molecules: pharmaco-chemical drug delivery strategies

Pharmaco-chemical strategies, such as making a drug more lipophilic ("lipidisation") or design of a BBB permeable pro-drug can be attractive, but often the pharmacological properties are lost by modification of the drug. Furthermore, "lipidisation" also enhances diffusion through other membranes, thereby increasing side effects. This results in a larger volume of distribution and therefore a lower concentration in blood. Therefore, "lipidisation" results in a minimal change in actual drug delivery to the brain (50).

4.2. Large drug molecules: invasive and disruptive strategies for brain drug delivery

Invasive brain drug delivery strategies, such as direct intracerebral injections of slow release products only allow local delivery (figure 7B). This may be attractive for drug delivery to brain tumours, but not for the administration of drugs against more widespread diseases. Another invasive method is intra-ventricular drug infusion, in which a drug is injected into the CSF of the ventricular organs. However, CSF is completely absorbed from the ventricular organs into the venous circulation (figure 7C).

As a result the infused drug has minimal access to the parenchyma by diffusion (51). In general, invasive strategies are not effective for drug delivery to the whole brain, but only to a localised part of the brain.

Drug delivery through BBB disruption by osmotic imbalance or vaso-active compounds has the disadvantage that the brain can be damaged permanently due to unwanted blood components entering the brain (52).

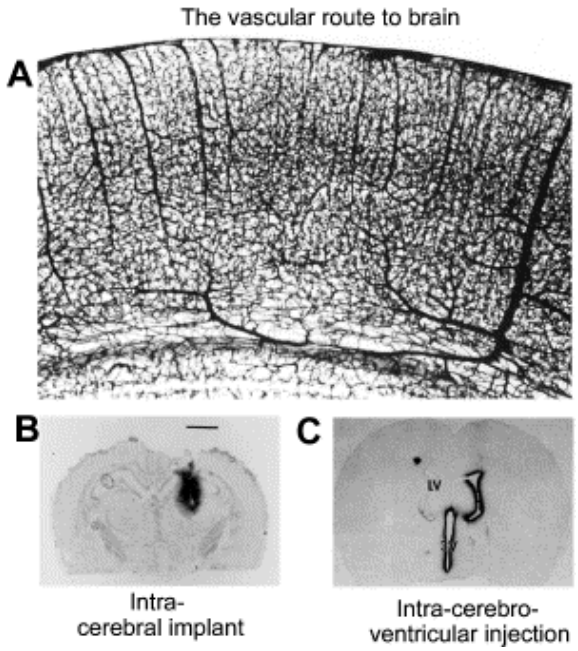


Figure 7: (**A**) Drug delivery via the vascular route enable widespread distribution of the drug to all cells within the brain. (**B**) Minimal diffusion of [^{125}I]-nerve-growth factor (NGF) after intracerebral implantation of a biodegradable polymer. (**C**) ICV injection of [^{125}I]- brain-derived neurotrophic factor (BDNF). The neurotrophin does not distribute into the brain beyond the ipsilateral ependymal surface. From Pardridge (1).

4.3. Physiological drug targeting strategies for brain drug delivery

Physiological drug delivery strategies aim to use endogenous transport mechanisms at the BBB, such as adsorptive-mediated, carrier-mediated or receptor mediated transcytosis. The advantage of the vascular route is the widespread diffusion of the infused drug across the whole brain (1) (figure 7A). This can be explained by the large surface area of the human BBB (approximately 20 m^2). In addition, approximately each neuron has its own brain capillary for oxygen supply as well as the supply of other nutrients. This means that the vascular route is a very promising one for drug targeting and delivery to the brain.

For small drug molecules drug delivery via carrier-mediated transcytosis is possible. Glycopeptide drugs, such as a glycosilated analogue of Met^5-enkephalin are transported

via the hexose transporter GLUT1 (21, 53). In addition, glycosilation of a linear opioid peptide resulted in an improved BBB transcytosis, as well as an improved metabolic stability (54). The best known example of carrier-mediated drug delivery is the transport of L-dopa, a precursor of the neurotransmitter dopamine, in the treatment of Parkinson's disease by the neutral amino acid carrier (16, 55). The disadvantage of carrier-mediated drug delivery is that a drug should mimic an endogenous nutrient (50).

Adsorptive-mediated transcytosis (AMT) is triggered by an electrostatic interaction and can transport larger drug molecules to the brain. The best known compound that is targeted to the brain via this mechanism is cationised albumin (21, 56). An example of a drug transported by AMT is Ebiratide, a synthetic peptide analogue of adrenocorticotropic hormone for the treatment of Alzheimer's disease (57, 58). Ebiratide is positively charged and is resistant to metabolism during the transcytosis across the BBB. AMT is not very specific, but the higher capacity of AMT, compared to receptor-mediated transcytosis, is a favourable property for the delivery of peptides to the brain (4, 21).

A more specific delivery of larger drug molecules or drug carrying particles to the brain can be reached through receptor-mediated transcytosis. Upon receptor-ligand internalisation clathrin-coated vesicles are formed (59). These clathrin-coated vesicles are approximately 120 nm in diameter (60). In this thesis the focus is on the TfR, but also the insulin receptor or the scavenger receptors at the BBB can be used for drug delivery. For the human insulin receptor a monoclonal antibody (HIRMAb) has been developed, which is active in both humans and Old World primates. Approximately 4% of the injected HIRMAb is delivered to the primate brain *in vivo* (61). In addition, the BBB permeability coefficient of the HIRMAb is nine-fold greater than of any other known vector, including vectors directed to the TfR (62). Furthermore, in addition to Tf, P97, or melano-transferrin is also used for drug targeting to the brain. P97 is not taken up via the TfR, but it is selective for drug targeting to the brain, probably via the low-density lipoprotein receptor-related protein (63). However, P97 also activates plasminogen, which could affect angiogenesis as well as blood clotting (64).

Much research has been performed towards a monoclonal antibody against the rat TfR (OX-26) as a targeting vector (49, 65). Furthermore, Fab fragments or small peptides have been developed to target the TfR and to induce receptor internalisation (66). The endogenous ligand, Tf, is also used for drug targeting (67-69). However, the

endogenous concentration of Tf in serum is already around 25 μM, therefore, competition for the TfR might be expected. Tf has been under investigation for the targeting of anti-tumour agents, and for the delivery of gene therapeutics, using polycation-based drug carriers. Furthermore, Tf has been used for the delivery of therapeutically active metals, such as manganese (important as trace element and in several enzymes, such as superoxide dismutase), gallium (as a radiodiagnostic agent) or ruthenium (as a potential anticancer therapy) (70).

4.4. Drug conjugates and liposomes for brain drug delivery

In contrast to pharmaco-chemical, invasive and disruptive strategies, physiological strategies include the application of the drug, a targeting vector and a linker strategy. This linker can either be a direct linker between the drug and the targeting vector, or the drug can be incorporated in a drug carrier, which is tagged with the targeting vector (figure 8). Since the scope of this thesis is on the possibilities for drug targeting to the TfR, this paragraph focuses on the endogenous ligand of the TfR, Tf, and the antibody against the TfR (OX-26) as targeting vectors. A selection of linker strategies will be discussed, focussing mainly on the larger biotechnology drugs, such as proteins and gene medicines.

Proteins can be directly linked to Tf or OX-26 via an avidin-biotin linker, in which a mono-biotinylated protein is conjugated with OX-26 containing a streptavidin or avidin moiety (figure 8A) (71). It is also possible to increase the distance between the drug and the targeting vector, by inserting a PEG-molecule between the biotin moiety and the protein drug (figure 8B). This increased distance reduces the steric hindrance by the OX-26 antibody and increases the possibilities for receptor recognition by the protein drug (72). In addition, proteins or antibodies can be modified at their N-terminus, without loosing receptor-recognition or drug effect. Agents that are used for N-terminus modification are N-succinimidyl S-acetylthioacetate (SATA, (73)), or 2-iminothiolane (Traut's reagent, (74)) to insert a thiol group. This thiol can react with another thiol to form a cleavable disulfide bond. Alternatively, the thiol group can react with a maleimide group to form a stable thio-ester bond.

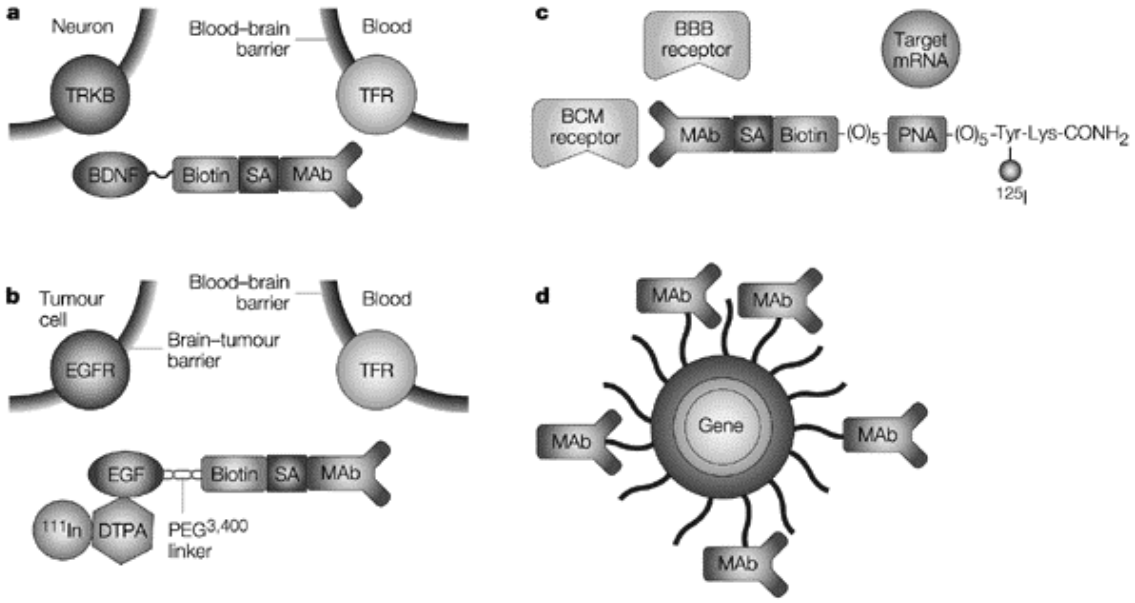


Figure 8: Structures of delivery vehicles for crossing the BBB. (**a**) A direct coupling of the targeting vector (Mab) to the drug (brain-derived neurotrophic factor, BDNF) via the avidin-biotin technology (**b**) Poly(ethyleneglycol) (PEG3,400) is placed between the epidermal growth factor (EGF) and the transport vector to release any steric hindrance of EGF binding to the EGF receptor. (**c**) Structure of an antisense radiopharmaceutical (polynucleic acid, PNA) that is coupled to the Mab to enable transport across both the BBB. (**d**) A double-stranded supercoiled plasmid DNA containing an exogenous gene that is packaged into the interior of an 85-nm liposome. The surface of the liposome is conjugated with ~2,000 strands of PEG, and the tips of 1–2% of the PEG strands are conjugated with a targeting MAb. From Pardridge (87).

Gene medicines, such as antisense mRNA, can also be coupled directly to OX-26 or Tf via the avidin-biotin technology (figure 8C) (75, 76). However, it is necessary to stabilise the mRNA to avoid degradation in serum. Gene medicines can also be incorporated in a drug carrier (figure 8D). This drug carrier can be conventional or sterically stabilised liposomes (77, 78), lipoplexes (79) or cationic amphiphiles (80). Incorporation into a drug carrier protects the drug against degradation in serum. In addition, it is not necessary to modify the drug, thereby preserving the drug properties. For drug targeting to the brain, liposomes are used, consisting of biodegradable phospholipids, which are not immunogenic (81). For prolonged circulation time *in vivo* and increased stability of the liposomes polyethylene glycol (PEG) can be added to the bilayer (82, 83). In general PEG with a molecular weight of 2000 is used at a

concentration of 5%, based on molar ratios. Tf or OX-26 can be coupled to the surface of the liposome, via a maleimide linker, which is attached to a lipid anchor. However, when PEG is attached as well, this "PEG-coat" causes steric hindrance for TfR recognition. Therefore, PEG has been modified with a maleimide group attached to its distal end, enabling Tf or OX-26 coupling (84).

Lipoplexes are formed by positively charged polymers, which can condens negatively charged DNA or mRNA (81, 85). These particles can also be stabilised by PEG. Synthetic amphiphiles are vesicles which are formed by non-biodegrable lipids. SAINT is a well known constituent of synthetic amphiphiles, together with DOPE (80, 86). These drug carriers are often used for transfection purposes, but with PEG their circulation time in the blood can be prolonged as well. Shedable PEG carrying colloidal systems are now under investigation, since the stabilised synthetic PEG-coated amphiphiles showed less transfection. Shedable PEG (PEG-ceramide) is released from the drug carrier in time, after which transfection of the target cells can take place (80, 86). For lipoplexes, as well as synthetic amphiphiles the PEG can be tagged with Tf or OX-26.

5. Summary

In summary, many drug targeting and drug delivery strategies for drug delivery to the brain have been developed. The research described in this thesis will focus on the TfR as a model transport system for drug delivery to the brain. The TfR is highly expressed at the BBB, but also on other cells in the body. Although drug targeting to the brain via the TfR is therefore not selective, it is effective (for review, see (1, 4)). The endogenous ligand, Tf, is used as a targeting vector for drug conjugates as well as liposomes. For *in vivo* applications the use of Tf is limited, since the Tf concentration in serum is high. However, the research described in this thesis uses a mechanistic approach to elucidate the efficacy of endocytosis of Tf-drug conjugates and Tf-tagged liposomes. The results of this research can be used to improve drug delivery to the brain.

6. References

1. Pardridge, W. M. (2002) Drug and gene delivery to the brain: the vascular route. *Neuron* **36** (4): 555-558
2. Pardridge, W. M. (1999) Blood-brain barrier biology and methodology. *J Neurovirol* **5** (6): 556-569
3. Begley, D. J. (1996) The blood-brain barrier: principles for targeting peptides and drugs to the central nervous system. *J Pharm Pharmacol* **48** (2): 136-146
4. Pardridge, W. M. (2001) Drug targeting, drug discovery, and brain drug development. In *Brain drug targeting - The future of brain drug development* (W. M. Pardridge, ed.), University Press, Cambridge, pp. 1-12
5. Ehrlich, P. (1885) *Das Sauerstoff-Bedurfnis des Organismus: eine farbenanalytische Studie*. Hirschwald, Berlin
6. Goldman, E. E. (1913) Vitalfarbung am Zentralnervensystem. *Abh. Preuss. Akad. Wiss. Phys. Math.* **K1** (1): 1-60
7. Rubin, L. L. and Staddon, J. M. (1999) The cell biology of the blood-brain barrier. *Annu Rev Neurosci* **22** 11-28
8. Pardridge, W. M. (1991) Overview of Blood-Brain Barrier Transport Biology and Experimental Methodologies. In *Peptide Drug Delivery to the Brain* (W. M. Pardridge, ed.), Raven Press, New York, pp. 52 - 98
9. Reese, T. S. and Karnovsky, M. J. (1967) Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J Cell Biol* **34** (1): 207-217
10. Brightman, M. W. and Reese, T. S. (1969) Junctions between intimately opposed cell membranes in the vertebrate brain. *J Cell Biol* **40** (3): 648-677
11. DeBault, L. E. and Cancilla, P. A. (1980) gamma-Glutamyl transpeptidase in isolated brain endothelial cells: induction by glial cells in vitro. *Science* **207** (4431): 653-655
12. Hendry, S. H., Jones, E. G. and Beinfeld, M. C. (1983) Cholecystokinin-immunoreactive neurons in rat and monkey cerebral cortex make symmetric synapses and have intimate associations with blood vessels. *Proc Natl Acad Sci U S A* **80** (8): 2400-2404
13. de Boer, A. G., van der Sandt, I. C. and Gaillard, P. J. (2003) The role of drug transporters at the blood-brain barrier. *Annu Rev Pharmacol Toxicol* **43** 629-656
14. Abbott, N. J. (1992) Comparative physiology of the blood-brain barrier. In *Physiology and pharmacology of the blood-brain barrier* (M. W. Bradbury, ed.), Springer-Verlag, Berlin, pp. 371-396.
15. Bradbury, M. W. (1979) *The concept of a blood-brain barrier*. John Wiley & Sons Ltd., Chichester
16. Pardridge, W. M. (1995) Transport of small molecules through the blood-brain barrier: biology and methodology. *Adv Drug Deliv Rev* **15** 5-36
17. Stein, W. D. (1967) *The movement of molecules across cell membranes*. Academic Press, New York
18. Lipinski, C. A., Lombardo, F., Dominy, B. W. and Feeney, P. J. (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* **46** (1-3): 3-26
19. Abraham, M. H., Chadha, H. S. and Mitchell, R. C. (1994) Hydrogen bonding. 33. Factors that influence the distribution of solutes between blood and brain. *J Pharm Sci* **83** (9): 1257-1268

20. Abbott, N. J. and Romero, I. A. (1996) Transporting therapeutics across the blood-brain barrier. *Mol Med Today* **2** (3): 106-113
21. Tsuji, A. and Tamai, I. I. (1999) Carrier-mediated or specialized transport of drugs across the blood-brain barrier. *Adv Drug Deliv Rev* **36** (2-3): 277-290
22. Tamai, I. and Tsuji, A. (2000) Transporter-mediated permeation of drugs across the blood-brain barrier. *J Pharm Sci* **89** (11): 1371-1388
23. Vorbrodt, A. W. (1989) Ultracytochemical characterization of anionic sites in the wall of brain capillaries. *J Neurocytol* **18** (3): 359-368
24. Bickel, U., Yoshikawa, T. and Pardridge, W. M. (2001) Delivery of peptides and proteins through the blood-brain barrier. *Adv Drug Deliv Rev* **46** (1-3): 247-279
25. Duffy, K. R. and Pardridge, W. M. (1987) Blood-brain barrier transcytosis of insulin in developing rabbits. *Brain Res* **420** (1): 32-38
26. Pardridge, W. M., Eisenberg, J. and Yang, J. (1987) Human blood-brain barrier transferrin receptor. *Metabolism* **36** (9): 892-895
27. Moos, T. and Morgan, E. H. (2000) Transferrin and transferrin receptor function in brain barrier systems. *Cell Mol Neurobiol* **20** (1): 77-95
28. Dehouck, B., Fenart, L., Dehouck, M. P., Pierce, A., Torpier, G. and Cecchelli, R. (1997) A new function for the LDL receptor: transcytosis of LDL across the blood-brain barrier. *J Cell Biol* **138** (4): 877-889
29. Bjorbaek, C., Elmquist, J. K., Michl, P., Ahima, R. S., van Bueren, A., McCall, A. L. and Flier, J. S. (1998) Expression of leptin receptor isoforms in rat brain microvessels. *Endocrinology* **139** (8): 3485-3491
30. Duffy, K. R., Pardridge, W. M. and Rosenfeld, R. G. (1988) Human blood-brain barrier insulin-like growth factor receptor. *Metabolism* **37** (2): 136-140
31. van der Sandt, I. C., de Boer, A. G. and Breimer, D. D. (2004) Implications of Pgp for the transport and distribution of drugs into the brain. In *Blood-spinal cord and brain barriers in health and disease* (H. S. Sharma and J. Wesman, eds.), Elsevier Academic Press, San Diego, pp. 63-72
32. Zhang, Y., Han, H., Elmquist, W. F. and Miller, D. W. (2000) Expression of various multidrug resistance-associated protein (MRP) homologues in brain microvessel endothelial cells. *Brain Res* **876** (1-2): 148-153
33. Lee, G., Dallas, S., Hong, M. and Bendayan, R. (2001) Drug transporters in the central nervous system: brain barriers and brain parenchyma considerations. *Pharmacol Rev* **53** (4): 569-596
34. Morgan, E. H. (1996) Iron metabolism and transport. In *Hepatology. A textbook of liver disease*, Vol. 1 (D. Zakin and T. D. Boyer, eds.), Saunders, Philadelphia, pp. 526-554
35. Ponka, P. and Lok, C. N. (1999) The transferrin receptor: role in health and disease. *Int J Biochem Cell Biol* **31** (10): 1111-1137
36. Huebers, H. A. and Finch, C. A. (1987) The physiology of transferrin and transferrin receptors. *Physiol Rev* **67** (2): 520-582
37. Kuhn, L. C. (1991) mRNA-protein interactions regulate critical pathways in cellular iron metabolism. *Br J Haematol* **79** (1): 1-5

38. Casey, J. L., Koeller, D. M., Ramin, V. C., Klausner, R. D. and Harford, J. B. (1989) Iron regulation of transferrin receptor mRNA levels requires iron- responsive elements and a rapid turnover determinant in the 3' untranslated region of the mRNA. *Embo J* **8** (12): 3693-3699
39. Trinder, D. and Baker, E. (2003) Transferrin receptor 2: a new molecule in iron metabolism. *Int J Biochem Cell Biol* **35** (3): 292-296
40. Kohgo, Y., Nishisato, T., Kondo, H., Tsushima, N., Niitsu, Y. and Urushizaki, I. (1986) Circulating transferrin receptor in human serum. *Br J Haematol* **64** (2): 277-281
41. Shih, Y. J., Baynes, R. D., Hudson, B. G., Flowers, C. H., Skikne, B. S. and Cook, J. D. (1990) Serum transferrin receptor is a truncated form of tissue receptor. *J Biol Chem* **265** (31): 19077-19081
42. Kohgo, Y., Torimoto, Y. and Kato, J. (2002) Transferrin receptor in tissue and serum: updated clinical significance of soluble receptor. *Int J Hematol* **76** (3): 213-218
43. Baker, E. and Morgan, E. H. (1994) Iron transport. In *Iron metabolism in health and disease* (Brock, ed.), Saunders, London, pp. 63-95
44. Li, H. and Qian, Z. M. (2002) Transferrin/transferrin receptor-mediated drug delivery. *Med Res Rev* **22** (3): 225-250
45. Moos, T. and Morgan, E. H. (2004) The significance of the mutated divalent metal transporter (DMT1) on iron transport into the Belgrade rat brain. *J Neurochem* **88** (1): 233-245
46. Qian, Z. M., Li, H., Sun, H. and Ho, K. (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharmacol Rev* **54** (4): 561-587
47. Huwyler, J. and Pardridge, W. M. (1998) Examination of blood-brain barrier transferrin receptor by confocal fluorescent microscopy of unfixed isolated rat brain capillaries. *J Neurochem* **70** (2): 883-886
48. Zhang, Y. and Pardridge, W. M. (2001) Rapid transferrin efflux from brain to blood across the blood-brain barrier. *J Neurochem* **76** (5): 1597-1600
49. Pardridge, W. M. (2003) Blood-brain barrier drug targeting: the future of brain drug development. *Mol Interv* **3** (2): 90-105, 151
50. Pardridge, W. M. (1999) Non-invasive drug delivery to the human brain using endogenous blood-brain barrier transport systems. *Pharm Sci Technol Today* **2** (2): 49-59
51. Aird, R. B. (1984) A study of intrathecal, cerebrospinal fluid-to-brain exchange. *Exp Neurol* **86** (2): 342-358
52. Miller, G. (2002) Drug targeting. Breaking down barriers. *Science* **297** (5584): 1116-1118
53. Polt, R., Porreca, F., Szabo, L. Z., Bilsky, E. J., Davis, P., Abbruscato, T. J., Davis, T. P., Harvath, R., Yamamura, H. I. and Hruby, V. J. (1994) Glycopeptide enkephalin analogues produce analgesia in mice: evidence for penetration of the blood-brain barrier. *Proc Natl Acad Sci U S A* **91** (15): 7114-7118
54. Egleton, R. D., Mitchell, S. A., Huber, J. D., Palian, M. M., Polt, R. and Davis, T. P. (2001) Improved blood-brain barrier penetration and enhanced analgesia of an opioid peptide by glycosylation. *J Pharmacol Exp Ther* **299** (3): 967-972
55. Wade, L. A. and Katzman, R. (1975) Rat brain regional uptake and decarboxylation of L-DOPA following carotid injection. *Am J Physiol* **228** (2): 352-359
56. Thole, M., Nobmanna, S., Huwyler, J., Bartmann, A. and Fricker, G. (2002) Uptake of cationized albumin coupled liposomes by cultured porcine brain microvessel endothelial cells and intact brain capillaries. *J Drug Target* **10** (4): 337-344

57. Terasaki, T., Takakuwa, S., Saheki, A., Moritani, S., Shimura, T., Tabata, S. and Tsuji, A. (1992) Absorptive-mediated endocytosis of an adrenocorticotropic hormone (ACTH) analogue, ebiratide, into the blood-brain barrier: studies with monolayers of primary cultured bovine brain capillary endothelial cells. *Pharm Res* **9** (4): 529-534
58. Shimura, T., Tabata, S. and Hayashi, S. (1991) Brain transfer of a new neuromodulating ACTH analog, ebiratide, in rats. *Peptides* **12** (3): 509-512
59. Gumbleton, M., Abulrob, A. G. and Campbell, L. (2000) Caveolae: an alternative membrane transport compartment. *Pharm Res* **17** (9): 1035-1048
60. Conner, S. D. and Schmid, S. L. (2003) Regulated portals of entry into the cell. *Nature* **422** (6927): 37-44
61. Pardridge, W. M., Kang, Y. S., Buciak, J. L. and Yang, J. (1995) Human insulin receptor monoclonal antibody undergoes high affinity binding to human brain capillaries in vitro and rapid transcytosis through the blood-brain barrier in vivo in the primate. *Pharm Res* **12** (6): 807-816
62. Wu, D., Yang, J. and Pardridge, W. M. (1997) Drug targeting of a peptide radiopharmaceutical through the primate blood-brain barrier in vivo with a monoclonal antibody to the human insulin receptor. *J Clin Invest* **100** (7): 1804-1812
63. Demeule, M., Poirier, J., Jodoin, J., Bertrand, Y., Desrosiers, R. R., Dagenais, C., Nguyen, T., Lanthier, J., Gabathuler, R., Kennard, M., Jefferies, W. A., Karkan, D., Tsai, S., Fenart, L., Cecchelli, R. and Beliveau, R. (2002) High transcytosis of melanotransferrin (P97) across the blood-brain barrier. *J Neurochem* **83** (4): 924-933
64. Demeule, M., Bertrand, Y., Michaud-Levesque, J., Jodoin, J., Rolland, Y., Gabathuler, R. and Beliveau, R. (2003) Regulation of plasminogen activation: a role for melanotransferrin (p97) in cell migration. *Blood* **102** (5): 1723-1731
65. Jefferies, W. A., Brandon, M. R., Hunt, S. V., Williams, A. F., Gatter, K. C. and Mason, D. Y. (1984) Transferrin receptor on endothelium of brain capillaries. *Nature* **312** (5990): 162-163
66. Lee, J. H., Engler, J. A., Collawn, J. F. and Moore, B. A. (2001) Receptor mediated uptake of peptides that bind the human transferrin receptor. *Eur J Biochem* **268** (7): 2004-2012
67. Eavarone, D. A., Yu, X. and Bellamkonda, R. V. (2000) Targeted drug delivery to C6 glioma by transferrin-coupled liposomes. *J Biomed Mater Res* **51** (1): 10-14
68. Voinea, M., Dragomir, E., Manduteanu, I. and Simionescu, M. (2002) Binding and uptake of transferrin-bound liposomes targeted to transferrin receptors of endothelial cells. *Vascul Pharmacol* **39** (1-2): 13-20
69. Widera, A., Bai, Y. and Shen, W. C. (2004) The transepithelial transport of a G-CSF-transferrin conjugate in Caco-2 cells and its myelopoietic effect in BDF1 mice. *Pharm Res* **21** (2): 278-284
70. Sun, H., Li, H. and Sadler, P. J. (1999) Transferrin as a metal ion mediator. *Chem Rev* **99** (9): 2817-2842
71. Bickel, U., Yoshikawa, T., Landaw, E. M., Faull, K. F. and Pardridge, W. M. (1993) Pharmacologic effects in vivo in brain by vector-mediated peptide drug delivery. *Proc Natl Acad Sci U S A* **90** (7): 2618-2622
72. Pardridge, W. M., Wu, D. and Sakane, T. (1998) Combined use of carboxyl-directed protein pegylation and vector-mediated blood-brain barrier drug delivery system optimizes brain uptake of brain-derived neurotrophic factor following intravenous administration. *Pharm Res* **15** (4): 576-582

73. Duncan, R. J., Weston, P. D. and Wrigglesworth, R. (1983) A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal Biochem* **132** (1): 68-73
74. Lasch, J., Niedermann, G., Bogdanov, A. A. and Torchilin, V. P. (1987) Thiolation of preformed liposomes with iminothiolane. *FEBS Lett* **214** (1): 13-16
75. Pardridge, W. M., Boado, R. J. and Kang, Y. S. (1995) Vector-mediated delivery of a polyamide ("peptide") nucleic acid analogue through the blood-brain barrier in vivo. *Proc Natl Acad Sci U S A* **92** (12): 5592-5596
76. Wu, D., Boado, R. J. and Pardridge, W. M. (1996) Pharmacokinetics and blood-brain barrier transport of [3H]-biotinylated phosphorothioate oligodeoxynucleotide conjugated to a vector-mediated drug delivery system. *J Pharmacol Exp Ther* **276** (1): 206-211
77. Willis, M. and Forssen, E. (1998) Ligand-targeted liposomes. *Adv Drug Deliv Rev* **29** (3): 249-271
78. Storm, G. and Crommelin, D. J. A. (1998) Liposomes: Quo Vadis? *PSTT* **1** (1): 19-31
79. Fenske, D. B., MacLachlan, I. and Cullis, P. R. (2001) Long-circulating vectors for the systemic delivery of genes. *Curr Opin Mol Ther* **3** (2): 153-158
80. Zuhorn, I. S. and Hoekstra, D. (2002) On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? *J Membr Biol* **189** (3): 167-179
81. Crommelin, D. J., Storm, G., Jiskoot, W., Stenekes, R., Mastrobattista, E. and Hennink, W. E. (2003) Nanotechnological approaches for the delivery of macromolecules. *J Control Release* **87** (1-3): 81-88
82. Schmidt, J., Metselaar, J. M., Wauben, M. H., Toyka, K. V., Storm, G. and Gold, R. (2003) Drug targeting by long-circulating liposomal glucocorticosteroids increases therapeutic efficacy in a model of multiple sclerosis. *Brain* **126** (Pt 8): 1895-1904
83. Shi, N., Boado, R. J. and Pardridge, W. M. (2001) Receptor-mediated gene targeting to tissues in vivo following intravenous administration of pegylated immunoliposomes. *Pharm Res* **18** (8): 1091-1095
84. Huwyler, J., Wu, D. and Pardridge, W. M. (1996) Brain drug delivery of small molecules using immunoliposomes. *Proc Natl Acad Sci U S A* **93** (24): 14164-14169
85. van Steenis, J. H., van Maarseveen, E. M., Verbaan, F. J., Verrijk, R., Crommelin, D. J., Storm, G. and Hennink, W. E. (2003) Preparation and characterization of folate-targeted PEG-coated pDMAEMA-based polyplexes. *J Control Release* **87** (1-3): 167-176
86. Shi, F., Nomden, A., Oberle, V., Engberts, J. B. and Hoekstra, D. (2001) Efficient cationic lipid-mediated delivery of antisense oligonucleotides into eukaryotic cells: down-regulation of the corticotropin-releasing factor receptor. *Nucleic Acids Res* **29** (10): 2079-2087
87. Pardridge, W. M. (2002) Drug and gene targeting to the brain with molecular Trojan horses. *Nat Rev Drug Discov* **1** (2): 131-139

Section 2

The transferrin receptor: Target Validation

Chapter 3

Characterisation and modulation of the transferrin receptor on brain capillary endothelial cells

Pharmaceutical Research (2004) 21 (5): 761 – 769

C.C. Visser, L.H. Voorwinden, D.J.A. Crommelin*, M. Danhof and A.G. de Boer

Leiden/Amsterdam Centre for Drug Research (LACDR), Leiden University, Division of Pharmacology, PO Box 9502, 2300 RA Leiden, The Netherlands

*Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), PO Box 80082, 3508 TB Utrecht, The Netherlands

Abstract

Drug targeting to the brain often focuses on the transferrin receptor (TfR), which is expressed at the blood-brain barrier (BBB). The objective of our research was to determine the expression level of the TfR on brain capillary endothelial cells (BCEC), as well as the endocytosis of ^{125}I-transferrin (^{125}I-Tf) by this receptor. Furthermore, the influence of iron, the iron scavenger deferoxamine mesylate (DFO), astrocytic factors, a GTP-ase inhibitor (tyrphostin-A8, T8), lipopolysaccharide (LPS), and the radical scavenger N-acetyl-L-cysteine (NAC) on the TfR expression was studied, to gain insight in the use and optimisation of the TfR for drug targeting to the brain.

Primary cultured bovine BCEC were incubated with ^{125}I-Tf at 4 °C (to determine binding) or at 37 °C (to determine endocytosis) in the absence or presence of the modulators. For full saturation curves in the absence or presence of iron or DFO, analysis was performed with a population approach using NONMEM, allowing us to estimate a single value for affinity (K_d, concentration of 50% receptor occupancy) and separate values for maximum receptor occupancy (B_{max}).

On BCEC *in vitro* the TfR is expressed extracellularly (B_{max} of 0.13 fmol/μg cell protein), but also has a large intracellular pool (total B_{max} of 1.37 fmol/μg cell protein), and is actively endocytosing Tf via clathrin-coated vesicles. At 4 °C a K_d of 2.38 μg/ml was found, while the K_d at 37 °C was 5.03 μg/ml. Furthermore, DFO is able to increase both the extracellular as well as the total binding capacity to 0.63 and 3.67 fmol/μg cell protein, respectively, while it had no influence on K_d. B_{max} at 37 °C after DFO pre-incubation was also increased from 0.90 to 2.31 fmol/μg cell protein. Other modulators had no significant influence on the TfR expression levels, although LPS increased cellular protein concentrations after 2h pre-incubation.

In conclusion, the TfR is expressed on BCEC and actively endocytoses Tf, making it a suitable target for drug delivery to the BBB and the CNS. DFO up-regulates the TfR expression level, which may influence targeting efficiency.

Introduction

The central nervous system (CNS) is protected by the blood-brain barrier (BBB) to maintain homeostasis. This barrier is situated at the brain capillaries and comprises endothelial cells, covered by the endfeet of astrocytes (1). Due to specific features, such as tight junctions between endothelial cells, a continuous basal membrane, low pinocytosis and a lack of fenestrae, in general only small lipophilic drugs can pass the BBB (2). Many drugs for disorders of the CNS do not meet these requirements. Therefore, special transport systems are necessary to transport these drugs to the brain (3, 4).

To avoid invasive strategies to enhance BBB permeability, such as osmotic BBB disruption, targeting to the CNS often is aimed at endogenous transporters (5), such as the insulin receptor (6), the LDL receptor (7), or the scavenger and HDL receptor (8). Our research focuses on the use of the transferrin receptor (TfR), which is an internalising receptor, for brain drug targeting. It has been shown that drugs targeted to this receptor with a monoclonal antibody-conjugate have an enhanced biological effect in the brain *in vivo* (9). The TfR is expressed on endothelial cells of the brain capillaries where it is involved in iron transport to the brain via receptor-mediated endocytosis of transferrin (Tf) (10). Furthermore, the TfR is also expressed on hepatocytes, erythrocytes, and on proliferating cells (11). The TfR is a 190 kDa transmembrane glycoprotein, consisting of 2 subunits which are linked by a disulfide bridge (10). A trypsin-sensitive site is present extracellularly and proteolytic cleavage at this site leads to the loss of Tf binding (11). Recently, a second TfR has been identified, TfR2 (12). TfR2 also delivers iron to cells, but it has a 25 times lower affinity for Tf, and the distribution of TfR2 is different from TfR. Our research focuses on the TfR.

The objective of this research is to characterize the TfR mechanistically, in the *in vitro* BBB model developed by Gaillard *et al* (13), for studying brain drug targeting to and through this receptor. Therefore, we have done extensive studies; not only to determine binding and association (i.e. a combination of binding and endocytosis), but also the extent and mechanism of endocytosis. Furthermore, the influence of several modulators on the TfR expression was studied to gain insight into the potential use of the TfR for drug targeting to the brain.

First, the binding characteristics and association of Tf by the TfR at the BBB *in vitro* were investigated, as well as the extent of endocytosis of Tf. The latter was done by removal of extracellularly bound ^{125}I-Tf by acid wash or proteolytic cleavage of the TfR. In addition, to investigate the mechanism of endocytosis, the influence of several inhibitors of endocytotic processes was studied. For this phenylarsine oxide (PhAsO) was used, as it inhibits the clathrin associated receptor-mediated pathway, which is associated with the TfR (14). N-ethylmaleimide (NEM) is used as a non-specific inhibitor (15) and indomethacin as an inhibitor of adsorptive-mediated endocytosis, associated with caveolae (15).

Subsequently, changes in TfR expression, following pre-incubation with several modulators, were studied. First the influence of iron on the binding and association of ^{125}I-Tf was investigated. The expression level of the TfR is mainly dependent on the iron concentration, as the mRNA of the TfR is stabilised by an iron-regulatory factor at low iron concentrations, but not at high concentrations (16, 17). Therefore, the TfR expression level was determined at high iron concentrations (18), by addition of an excess of $FeCl_3$, and at low iron concentrations by addition of the iron scavenger deferoxamine mesylate (DFO) (19). Furthermore, the influence of astrocytes on TfR expression and association was estimated, as it is known that the secretion of astrocytic factors induce and maintain many BBB properties of the BCEC (2). For the purpose of validating BBB- or brain drug targeting models it was highly relevant to determine the level of TfR expression in the absence or presence of astrocytic factors. Recently, it was shown that the GTP-ase inhibitor tyrphostin-A8 (T8) could increase the transcytosis of Tf-conjugates in Caco-2 cells (20). Therefore, we investigated the influence of T8 on the binding and endocytosis of ^{125}I-Tf. Finally, BCEC were stimulated with lipopolysaccharide (LPS) to study TfR expression under inflammatory disease conditions. The effect of LPS was determined after a short incubation (2 h), during which acute phase effects occur, and after 24 h incubation, to assess the long-term effects. In addition, BCEC were pre-incubated with the radical scavenger N-acetyl-L-cysteine (NAC), as it was shown that NAC could prevent the down-regulation of TfR expression by free radicals (21).

Experimental

Cell Culture

Primary brain capillary endothelial cells (BCEC) were cultured from isolated bovine brain capillaries as described before (13). Briefly, brain capillaries were seeded in type IV collagen and fibronectin-coated plastic culture flasks and cultured in a 1:1 mixture of DMEM+S (containing 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, non essential amino acids and 10% fetal calf serum) and astrocyte-conditioned medium (ACM), supplemented with 125 μg/ml heparin (DMEM+ACM) at 37 °C, 10% CO_2 for 4 - 5 days. At 70 % confluency the BCEC were passaged with trypsin-EDTA and seeded into a type IV collagen coated 48 wells plate at a density of 30.000 cells/well. BCEC were cultured in the same medium at 37 °C, 10% CO_2 for 5 days. Astrocyte conditioned medium (ACM) was obtained according to the method described by Gaillard *et al* (13).

Preparation of radiolabeled transferrin

Bovine Tf was iodinated using Iodogen®, as described before (22), with a few modifications. 200 μg Tf (2.5 mg/ml in 1.5 M Tris-HCl, pH 8.5) was added to 0.25 mCi [^{125}I]Na in an Iodogen® (10 μg) precoated tube and incubated for 30 minutes at 4 °C. After separation on a Sephadex G-25 column, the labelled Tf was further purified by extensive dialysis (at least 48 h, 4 changes of buffer) in phosphate buffered saline (PBS, pH 7.4) at 4 °C. The labelled Tf had a specific activity of 278 ± 199 $*10^3$ cpm/μg and contained < 3 % free ^{125}I (determined by precipitation with 10% (w/v) trichloroacetic acid). ^{125}I-Tf was stored at 4 °C and used within 2 weeks.

Determination of cell associated transferrin (general)

BCEC were checked under the microscope for confluency and morphology (spindle shape when confluent) (13). One hour prior to the experiment the medium was changed to DMEM to deplete the cells from endogenous Tf. Subsequently, BCEC were incubated with ^{125}I-Tf in a concentration range of 0.25 - 12 μg/ml (full saturation approach), or with a fixed concentration of 8 μg/ml ^{125}I-Tf in 100 μl PBS. Incubation was performed at 4 °C (to determine binding) for 2 h or at 37 °C (to determine association, which is a combination of binding and endocytosis) for 1 h. For the determination of the total receptor expression level 0.5 % (w/v) saponin, 1 mM PMSF and 1 μg/ml leupeptin were added to permeabilise the cellular membranes (23). After incubation, BCEC were washed 6 times with 0.5 ml ice-cold PBS and solubilised with 1 M NaOH (750 μl) to measure radioactivity (gamma-counter) or the cellular protein content (Bio-Rad DC protein assay).

Endocytosis experiments

To determine the extent of endocytosis, BCEC were incubated with 8 μg/ml ^{125}I-Tf in 100 μl PBS at 4 °C for 2 h or at 37 °C for 1 h, and rinsed twice with 0.5 ml ice-cold PBS. Thereafter, BCEC were incubated with 0.5 ml citric acid/phosphate buffer pH 5.0 (modification of (24)) for 10 min on ice or with 0.5 ml trypsin (0.25 mg/ml) for 30 min on ice (25). After acid wash, cells were quickly washed twice with citric acid/phosphate buffer and three times with PBS before solubilisation with NaOH. After trypsinisation cells were transferred to a tube containing DMEM with 10 % fetal calf serum and centrifuged for 5 min, at 400 G. BCEC were washed twice with PBS, before determination of the remaining cell-associated activity.

For the inhibition studies BCEC were pre-incubated for 10 min with PhAsO (10 μM), NEM (1 mM), or indomethacin (50 μg/ml). Concentrations and pre-incubation times were modified from literature (14, 15). Subsequently, BCEC were incubated with 8 μg/ml ^{125}I-Tf in 100 μl PBS in the presence of the inhibitors at 4 °C for 2 h or at 37 °C for 1 h. BCEC were washed and solubilised as described previously.

Modulation by iron, astrocytic factors and Tyrphostin A8

To determine the influence of iron, BCEC were pre-incubated for 24 h with 1 mM DFO or $FeCl_3$ before estimating binding and association of ^{125}I-Tf using the full saturation approach. Association is a combination of binding and endocytosis, since we have not discriminated between those, unless specified.

To determine the influence of astrocytic factors on the TfR expression level, BCEC were cultured for 5 days in either the normal medium, which is a 1:1 mixture of DMEM+S and ACM, supplemented with 125 μg/ml heparin (DMEM+ACM), or in DMEM+S supplemented with 125 μg/ml heparin (DMEM+hep) or in DMEM+S alone (DMEM+S). Iron and Tf concentrations in ACM and DMEM+S were determined by a colorimetric assay on a fully automated Hitachi 911 (Hitachi, Tokyo, Japan). Coefficients of variation of these assays are below 3 %.

To determine the influence of T8 on the endocytosis of Tf, BCEC were pre-incubated for 10 min with 0.125 – 0.5 mM T8. Subsequently, binding and association of ^{125}I-Tf were determined in the presence or absence of T8.

Inflammatory disease conditions

BCEC were pre-incubated with 100 ng/ml LPS for 2 or 24 h before the binding and association of ^{125}I-Tf were assessed. The effect of NAC was determined by an 1 h or an overnight (16 – 17h) pre-incubation with 10 mM NAC, followed by a pre-incubation with LPS for 2 h. Concentrations are from Gaillard *et al* (26), who showed that 100 ng/ml LPS increases the permeability of the BBB *in vitro* after 2 h. This effect was reversed by an overnight pre-incubation with 10 mM NAC.

Data analysis

In all experiments total binding was corrected for non-specific binding, which was determined in the presence of 500-fold excess of unlabeled Tf. All data are presented as the means of at least 3 individual experiments, performed in triplicate. Cpm values were corrected for the specific activity of the batch ^{125}I-Tf used for the experiment, as well as for the cellular protein content.

Full saturation experiments were analysed with a population approach using the conventional first order method implemented in NONMEM (Version V, NONMEM project group, University of California, San Francisco, USA). A user-defined model for a one-site binding approach, where $B = B_{max}*[C]/(K_d+[C])$, was implemented. In this equation B is the specific binding, B_{max} the maximal receptor occupancy, C is the concentration and K_d is the concentration at which 50 % receptor occupancy occurs. By using this population approach it was possible to estimate a single K_d value for all binding experiments and different B_{max} values for the total and extracellular expression level in control situation, or after pre-incubation with DFO or $FeCl_3$. K_d and B_{max} values are estimated for both 4 °C and 37 °C. B_{max} values at 37 °C represent the maximal receptor occupancy as a combination of binding and endocytosis. Intra-individual residual variation was determined using a proportional error model and model selection was based on the parameter estimates, parameter correlations, and their confidence intervals. Goodness-of-fit was analysed by visual inspection, as well as by the minimum value of the objective function provided by NONMEM.

Statistical analysis was performed by one-way ANOVA (Tukey-Kramer multiple comparison post-test) and the student's t-test, using GraphPad InStat version 3.00 (GraphPad Software, San Diego, California, USA).

Materials

Culture flasks were obtained from Greiner (Alphen a/d Rijn, the Netherlands) and 48 wells plates from Corning Costar (Cambridge, MA, USA). PBS, DMEM, supplements and fetal calf serum were purchased from BioWhittaker Europe (Verviers, Belgium). Type IV collagen, heparin, trypsin-EDTA, endothelial cell trypsin, Iodogen®, saponin, phenylmethylsulfonylfluoride (PMSF), NEM, PhAsO, indomethacin, LPS, DFO and $FeCl_3 \cdot 6\ H_20$ were obtained from Sigma (Zwijndrecht, the Netherlands), fibronectin from Boehringer Mannheim (Almere, the Netherlands) and leupeptin from Molecular Probes (Leiden, the Netherlands). Bovine holo-transferrin, T8 and NAC were purchased from ICN Pharmaceuticals (Zoetermeer, the Netherlands) and Bio-Rad DC protein assay reagents from Bio-Rad Laboratories (Veenendaal, the Netherlands). Citric acid monohydrate and trichloricacetic acid (TCA) were obtained from J.T. Baker (Deventer, the Netherlands) and di-sodium hydrogen phosphate dihydrate from Merck (Amsterdam, the Netherlands). Sephadex-G25 coarse and [^{125}I]Na were purchased from Amersham Pharmacia Biotech (Roosendaal, the Netherlands).

Results

Full saturation binding studies

TfR expression was determined by incubating the BCEC with 0.25 -12 μg/ml ^{125}I-Tf for 2 h at 4 °C. At a concentration of approximately 8 μg/ml ^{125}I-Tf full saturation of the TfR expressed on BCEC was observed, as is shown in figure 1A and B. Using the population approach a single unique value of the K_d was estimated, which was 2.38 ± 0.32 μg/ml, while separate B_{max} values were obtained for total and extracellular TfR expression. These were 1.37 ± 0.11 and 0.13 + 0.02 fmol/μg protein (table I), respectively, indicating that approximately 90 % of the TfRs is present in a large intracellular pool. After incubation of BCEC with ^{125}I-Tf at 37 °C a K_d of 5.03 ± 0.50 μg/ml was found, while B_{max} at 37 °C was estimated at 0.90 ± 0.06 fmol/μg protein (table I).

After pre-incubation with 1 mM DFO the total and the extracellular TfR expression were increased to 3.68 ± 0.48 and 0.63 ± 0.04 fmol/μg protein, respectively (figure 1A and B, table I). Pre-incubation with $FeCl_3$ did not change the total TfR expression level (1.64 ± 0.28 fmol/μg protein), while the B_{max} for the extracellular TfR expression level was too low to detect.

Experiments at 37 °C showed a 2-fold increase in B_{max} to 2.31 ± 0.14 fmol/μg protein after DFO pre-incubation, while after pre-incubation with $FeCl_3$ a B_{max} of 0.56 ± 0.04 fmol/μg protein was observed (figure 1C, table I).

Endocytosis

To show that the TfR is actively endocytosing its ligand, extracellularly bound ligand was removed by either washing with a citric acid buffer or by removal of the extracellular part of the TfR by trypsin cleavage. At 37 °C 0.05 – 0.06 ng Tf/μg cell protein remained cell-associated after acid wash or trypsin cleavage (figure 2), which is 70 - 80 % of the total amount of cell-associated Tf (0.07 ng Tf/μg cell protein). After binding of ^{125}I-Tf at 4 °C and trypsin cleavage the cell-associated Tf was of the level of non-specific binding (0.003 ng Tf/μg cell protein, $P < 0.01$ vs total), while after acid

wash approximately 50% of the extracellular bound Tf was still present (0.007 ng Tf/ µg cell protein).

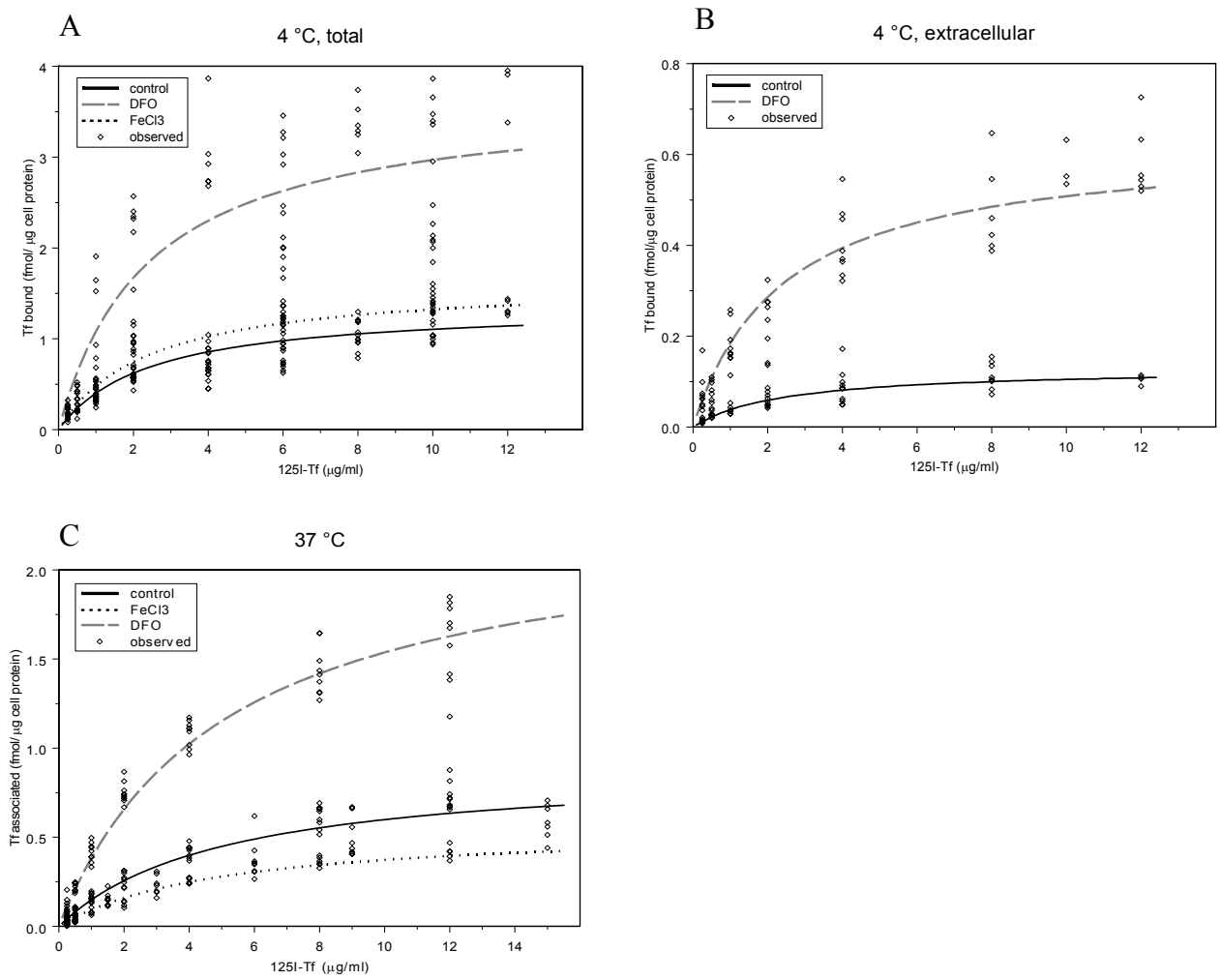


Figure 1: Total (**A**) and extracellular binding (**B**) of ^{125}I-Tf to the TfR, determined at 4 °C, and association (**C**) of ^{125}I-Tf by the TfR, determined at 37 °C, on primary cultured BCEC in the presence of DFO or $FeCl_3$ (1 mM). Data represented are the curves of specific binding, from at least 3 separate experiments performed in triplicate.

Several inhibitors were used to study the mechanism of the endocytotic process. BCEC were incubated with 8 µg/ml ^{125}I-Tf at 4 °C for 2 h or at 37 °C for 1 h in the absence or presence of PhAsO, an inhibitor of clathrin-mediated endocytosis, NEM, an inhibitor of most endocytotic processes or indomethacin, an inhibitor of caveolae mediated transport (figure 3). Total cell-associated Tf was 0.07 ng/µg cell protein,

PhAsO and NEM inhibited the endocytosis of ^{125}I-Tf almost completely to 0.003 and 0.007 ng Tf/μg cell protein, respectively. PhAsO and NEM had no effect on the binding of ^{125}I-Tf. Indomethacin had no significant effect on either the binding nor the endocytosis of ^{125}I-Tf (figure 3).

Table I: Total and extracellular expression, determined at 4 °C, of the TfR on primary cultured BCEC and the association of Tf, determined at 37 °C in the presence of DFO or $FeCl_3$ (1 mM). Data were analysed using NONMEM, K_d was estimated 2.4 ± 0.3 μg/ml and 5.0 ± 0.5 μg/ml for expression and association, respectively. Values for B_{max} are summarised in the table as mean (95 % confidence interval, CI). Intra-individual residual variation was determined with a proportional error model and was 10 % for expression levels and 9 % for association.

	B_{max} (fmol/μg cell protein)		
	total TfR expression	extracellular TfR expression	association
control	1.37	0.13	0.90
(CI)	(1.15 – 1.59)	(0.10 – 0.16)	(0.78 – 1.01)
DFO	3.68	0.63	2.31
(CI)	(2.73 – 4.63)	(0.55 – 0.71)	(2.03 – 2.59)
$FeCl_3$	1.64	not detectable	0.56
(CI)	(1.09 – 2.19)		(0.49 – 0.63)

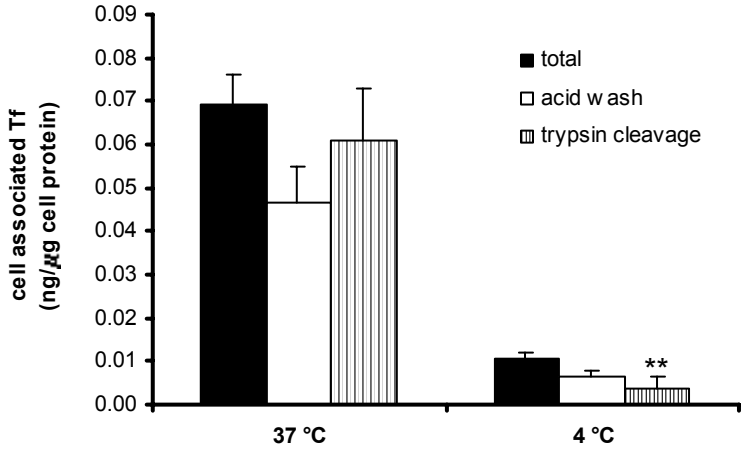


Figure 2: Cell-associated ^{125}I-Tf at 37 °C was reduced 20 – 30 % after acid wash (citric acid, pH 5) or trypsin cleavage (0.25 mg/ml trypsin), indicating that 70 – 80 % of the ^{125}I-Tf was endocytosed. At 4 °C the binding of ^{125}I-Tf is reduced to the level of non-specific binding after trypsin cleavage, indicating that no radioligand was endocytosed. Data are represented as mean ± s.d., one way ANOVA shows no difference between groups at 37 °C, but at 4 °C there is a difference; ** $P < 0.01$ (trypsin cleavage vs total; 4°C) Tukey-Kramer multiple comparison post-test.

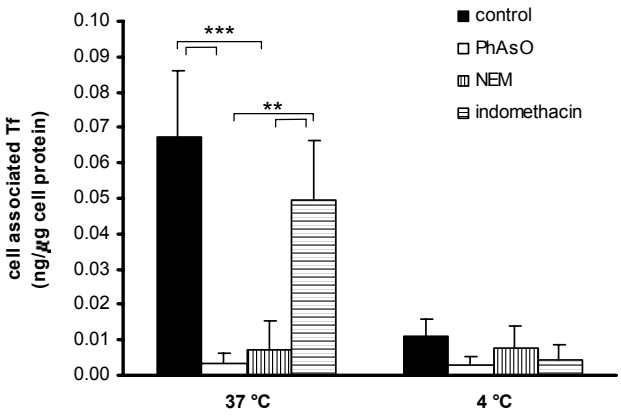


Figure 3: Endocytosis, determined at 37 °C, was inhibited after incubation with PhAsO (10 µM) and NEM (1 mM), but not after incubation with indomethacin (50 µg/ml), indicating that clathrin-mediated endocytosis is involved. At 4 °C non of these inhibitors had an effect on the extracellular binding of ^{125}I-Tf. Data represented are mean ± s.d., one-way ANOVA shows a difference between groups at 37 °C, but not at 4 °C; *** $P < 0.001$ (PhAsO, NEM vs control; 37 °C) ** $P < 0.01$ (PhAsO, NEM vs indomethacin; 37 °C), Tukey-Kramer multiple comparison post-test.

Modulation by astrocytic factors or Tyrphostin A8

BCEC were cultured for 5 days in DMEM+ACM, DMEM+hep or DMEM+S. After removal of endogenous Tf, by pre-incubation with DMEM-S, BCEC were incubated with 8 µg/ml ^{125}I-Tf for 2 h at 4 °C or for 1 h at 37 °C. No significant differences in total or extracellular TfR expression level were obtained after culturing BCEC in DMEM+hep (0.04 ± 0.01 and 0.01 ± 0.004 ng Tf/µg cell protein, for total and extracellular TfR expression, respectively) or DMEM+S (0.05 ± 0.02 and 0.01 ± 0.002 ng Tf/µg cell protein, for total and extracellular TfR expression, respectively), compared to the control situation DMEM+ACM (0.04 ± 0.01 and 0.01 ± 0.005 ng Tf/µg cell protein, for total and extracellular TfR expression, respectively). The association experiments at 37 °C showed a similar profile. After culturing BCEC in DMEM+hep or DMEM+S association was 0.06 ± 0.03 or 0.07 ± 0.03 ng Tf/µg cell protein, respectively. After culturing BCEC in DMEM+ACM association was 0.06 ± 0.02 ng Tf/µg cell protein.

To study the effect of T8, cells were pre-incubated with 0.25 mM T8 for 10 min. This had no effect on the binding and association of ^{125}I-Tf. After 1 h incubation with the radioligand at 37 °C, 0.045 ± 0.005 ng Tf/µg cell protein was associated, which was not different from the control (0.046 ± 0.012 ng Tf/µg cell protein). The extracellular binding was also not changed. Increasing the concentration T8 to 0.5 mM seems to downregulate the association of Tf, as only 0.031 ± 0.006 ng Tf/µg cell protein was

associated, though this is not statistically significant. Increasing the duration of the pre-incubation to 1 h did not change the effect of T8.

Inflammatory disease conditions

After pre-incubation of BCEC with LPS for 2 or 24 h the total TfR expression level was decreased from 0.14 ± 0.03 ng Tf/μg cell protein to 0.07 ± 0.01 ng Tf/μg cell protein after 2 h ($P < 0.01$) and to 0.10 ± 0.03 ng Tf/μg cell protein after 24 h (not significant). For the extracellular TfR expression, as well as for the association experiments at 37 °C a similar effect was found (figure 4 A and B). Pre-incubation of BCEC with 10 mM NAC for 1 h did not change the TfR expression level (0.10 ± 0.02 and 0.01 ± 0.002 ng Tf/μg cell protein for total and extracellular TfR, respectively) or association (0.05 ± 0.01 ng Tf/μg cell protein), but it did counteract the down-regulation of the TfR expression by a 2 h LPS pre-incubation when expressed in ng cell-associated Tf per μg cell protein (figure 4 A and B).

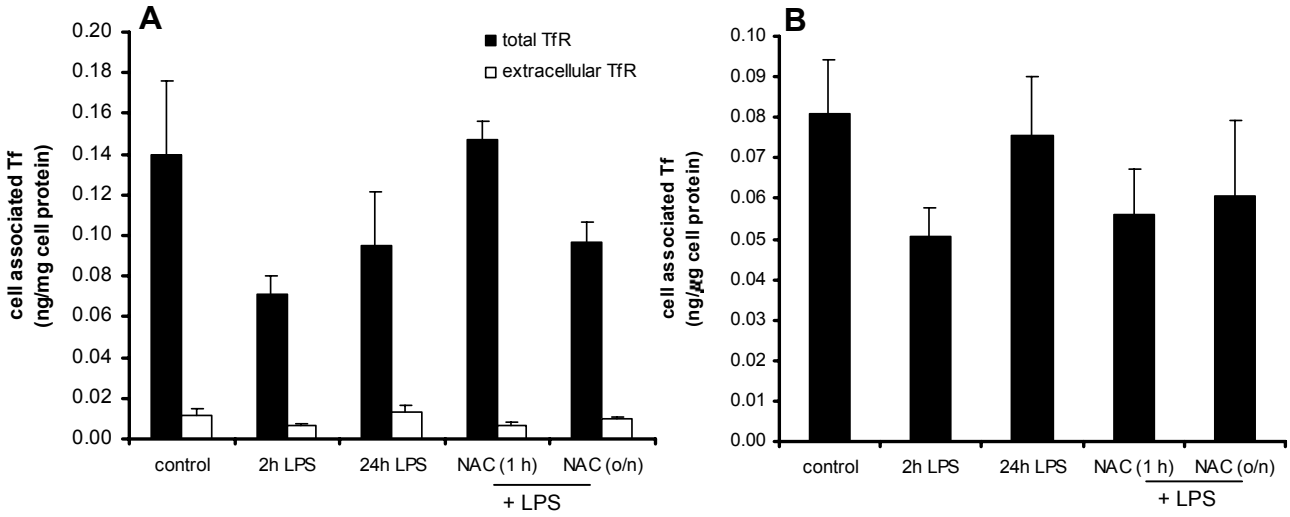


Figure 4: Total and extracellular binding (**A**) of ^{125}I-Tf to the TfR, determined at 4 °C, and association (**B**) of ^{125}I-Tf by the TfR, determined at 37 °C, after pre-incubation with 100 ng/ml LPS for 2 or 24 h, or after combined pre-incubation with 10 mM NAC (1 h or overnight), followed by LPS pre-incubation (2 h, 100 ng/ml). Data represented are the specific binding corrected for the cellular protein levels, from at least 3 separate experiments performed in triplicate. Statistical analysis was performed with one-way ANOVA (differences were found between groups at the total TfR level and at 37 °C, but not at the extracellular TfR expression level); * $P < 0.05$, ** $P < 0.01$ between groups, Tukey-Kramer multiple comparison post-test.

Increasing the pre-incubation time of NAC to 16 h (overnight) also had no effect on the TfR expression level and endocytosis, but it did prevent the down regulation of the TfR after 2 h LPS pre-incubation. However, no changes in total and extracellular TfR expression levels and association were observed after pre-incubation with LPS when the data were expressed in ng cell-associated Tf (figure 5 A and B). Interestingly, the cellular protein levels were increased after 2 h pre-incubation with LPS (table II), thereby decreasing the TfR expression level when expressed in ng cell-associated Tf per μg cell protein. Pre-incubation with NAC prevented the up-regulation of cellular protein and, thereby the apparent downregulation of TfR when expressed in ng cell associated Tf per μg cell protein.

Table II: Cellular protein levels after pre-incubation with 100 ng/ml LPS for 2 or 24 h, or after combined pre-incubation with 10 mM NAC (1 h or overnight (on)) and 100 ng/ml LPS (2 h). Data are mean ± s.d. from at least 3 experiments in triplicate.

	cellular protein levels (μg/well)		
	4 °C	4 °C + saponin	37 °C
control	22.9 ± 5.3	7.2 ± 3.0	21.3 ± 4.6
LPS (2 h)	28.2 ± 3.7	11.9* ± 2.3	31.6* ± 4.9
LPS (24 h)	23.4 ± 2.1	8.6 ± 2.9	24.8 ± 7.0
NAC (1 h) + LPS (2 h)	25.3 ± 1.9	9.0 ± 0.8	26.0 ± 4.0
NAC (on) + LPS (2 h)	24.5 ± 1.4	6.0 ± 1.5	24.9 ± 3.3

*$P < 0.05$, LPS (2 h) vs control (4 °C + saponin and 37 °C) and LPS (2 h) vs NAC (on) + LPS (4 °C + saponin), ANOVA analysis with Tukey-Kramer multiple comparisons post-test

Discussion

The passive transport of hydrophilic drugs to the CNS is limited by the BBB. This can be overcome by targeting drugs to transporters at the BBB. In addition, it has been shown that delivery of an exogenous gene, with liposomes targeted to the TfR on the BBB, exerts an effect in the brain *in vivo* (9). Therefore, this investigation focuses on the binding and internalisation of Tf by the TfR, expressed on bovine BCEC, which are

cultured in the presence of astrocytic factors. Furthermore, the influence of certain modulators and inflammatory disease conditions on the TfR expression and endocytosis was determined.

Binding studies at 4 °C showed a B_{max} of 0.13 ± 0.02 fmol/μg protein and a K_d of 2.38 ± 0.32 μg/ml for BCEC, cultured in the presence of astrocytic factors (figure 1A and B, table I). This K_d corresponds to approximately 30 nM, which is consistent with the 10 – 75 nM range found in literature (22, 23). It was possible to fully saturate the TfR after 2 h incubation with Tf concentrations up to 12 μg/ml. Binding experiments at 4 °C were carried out for 2 h, since association and dissociation of ^{125}I-Tf had reached equilibrium at 2 h (data not shown). Permeabilising the cells with saponin changed the observed B_{max} to 1.37 ± 0.11 fmol/μg protein (table I). This shows that approximately 90 % of the TfR is stored in an intracellular pool. This is consistent with observations by van Gelder *et al* (27) and Raub and Newton (23), albeit that a higher total TfR expression level was observed in the latter studies. This might be due to differences in isolation and cell culture procedures, as they isolated BCEC directly and not brain capillaries from which BCEC were grown (13). Furthermore, in the latter investigations BCEC were not cultured in medium containing astrocytic factors. From literature (1) it is known that the endfeet of astrocytes play an important role in inducing and maintaining BBB characteristics, such as tight junctions between endothelial cells and expression of certain transporters. However, due to high non-specific binding of the iodinated Tf, it was not possible to perform binding studies in our *in vitro* BBB model, which is a co-culture of bovine BCEC and rat astrocytes (13). Therefore, in the present investigation brain capillaries and BCEC were cultured in medium containing astrocytic factors.

By using a population approach it was possible to fit all saturation experiments simultaneously, thereby obtaining a single unique estimate of the affinity constant K_d and separate B_{max} values for all situations. In order to study the modulation of the TfR by iron, we pre-incubated BCEC with either $FeCl_3$ or with DFO. DFO is an iron scavenger, which appears to distribute into both intra- and extracellular spaces (19). Incubation with DFO resulted into an increase in B_{max} for both the total (3.68 ± 0.48 fmol/μg cell protein) as well as the extracellular (0.63 ± 0.04 fmol/μg cell protein) TfR expression level (figure 1A and B, table I). Interestingly, the extracellular TfR expression is increased 4.8 fold, while the total TfR expression is only increased 2.7 fold. Thus, the extracellular increase is not only due to synthesis of new receptors, but also to a shift

towards the outside of the BCEC. These experiments show that DFO increases the expression of the TfR, which could be useful for improved drug targeting to this receptor. DFO (desferal®) is currently used for treatment of (transfusional) iron overload, but it has many side effects (28). *In vivo* upregulation of the TfR may still be possible when new iron chelators are developed.

Addition of an excess of iron did not change the total TfR expression level, but at the extracellular TfR level it was not possible to detect a specific signal. This might be due to the disappearance of the TfR from the cell surface.

Experiments, performed at 37 °C, showed a B_{max} of 0.90 ± 0.06 fmol/μg protein, which is the maximal receptor occupancy as a combination of binding and endocytosis, and a K_d of 5.03 ± 0.50 μg/ml (figure 1C, table I). Incubation time was set to 1 h, since equilibrium between association and dissociation of ^{125}I-Tf was reached (data not shown). Pre-incubation with DFO resulted in a 2.6 fold increase of B_{max} at 37 °C, while pre-incubation with $FeCl_3$ resulted in a 1.6 fold decrease (figure 1C, table I). In these full saturation experiments we have not discriminated between internalised and extracellularly bound ^{125}I-Tf, nor have we investigated the retro-endocytosis of apo-Tf. Zhang and Pardridge (29) have shown that apo-Tf is rapidly effluxed from the brain. However, this does not necessarily suggest that the TfR is not suitable for drug targeting, since it is not investigated what happens with a Tf-drug conjugate or with Tf-tagged liposomes. In addition, it has been demonstrated that drug and gene transfer to the brain was successful following targeting to the TfR (9). The changes in B_{max} at 37 °C are consistent with the change that was found in extracellular TfR expression levels at 4 °C. Increasing the concentrations DFO and iron to 10 mM did not have an additional effect on the binding or endocytosis of ^{125}I-Tf.

In the analysis of these full saturation data, we initially estimated separate values for K_d. For experiments performed at 4 °C K_d values for extracellular binding were 3.2 ± 1.0 μg/ml and 4.5 ± 1.0 μg/ml for control and DFO, respectively, while for the total binding K_d values of 2.2 ± 0.5, 3.2 ± 0.9 and 3.0 ± 0.5 μg/ml were obtained for control, DFO and $FeCl_3$, respectively. Association experiments at 37 °C showed K_d values of 5.2 ± 0.9, 4.2 ± 0.8 and 5.5 ± 0.7 μg/ml for control, DFO and FeCl3, respectively. Statistical analysis has showed no difference between the K_d values for the binding or between the K_d values for the association. Therefore, a population approach was used, yielding a greater statistical power to determine the differences between up- and

downregulation of the TfR. Incorporation of inter-occasion variability in the modelling was considered. However, this did not contribute to the goodness of fit, since the triplicate measurements showed a very small inter-occasion variability. Several features of the stochastic model were investigated, which included inter-individual errors on all or some parameters. Furthermore, in the structural model the incorporation of a Hill factor was investigated. Based on the individual and population predictions, fitted through the data-points, the intra-individual residual variation, and the minimal value of the objective function, we concluded that addition of inter-individual variability on one or more parameters did not improve the goodness-of-fit. Incorporation of a Hill slope did not improve the fit either. Since it is known from literature that the binding of Tf to its receptor is 1:1 it is indeed justified to assume that the Hill slope is 1.

To further study the extent and mechanism of endocytosis of Tf by acid wash, trypsin cleavage or with inhibitors, a concentration of 8 μg/ml ^{125}I-Tf was used, as full saturation of the TfR is reached at this concentration. After acid wash or trypsin cleavage 70 - 80 % of the added ^{125}I-Tf remained cell-associated at 37 °C (figure 2). This was not significantly different from the total association, indicating that the TfR is actively endocytosing most of its ligand. At 4 °C trypsin cleavage removed ^{125}I-Tf to the level of non-specific binding, while with acid wash approximately 50% of the added Tf was removed. This rather inefficient removal might be due to the relatively high pH (pH 5.0) used for acid wash (24). Mostly, acid wash (30, 31) is performed at pH 3, but under those circumstances most of the BCEC are damaged in our experiments.

To study the process, by which the TfR is endocytosing Tf, several inhibitors were used, as is shown in figure 3. PhAsO and NEM inhibited the endocytosis of ^{125}I-Tf almost completely, while indomethacin had no significant effect. None of these inhibitors had an effect on the binding of ^{125}I-Tf, indicating that they did not alter the binding properties of Tf to the TfR. PhAsO is known to inhibit clathrin-mediated internalisation, which is associated with receptor-mediated endocytosis (14, 32). In this respect it was used to examine the contribution of receptor mediated endocytosis to the total intracellular uptake of Tf (31). NEM is a non-specific inhibitor of endocytotic processes, as it binds to a NEM-sensitive factor, which is responsible for the budding and fusion of a wide range of vesicles (15). Indomethacin reduces the number of plasmalemmal caveolae (15), thereby inhibiting caveolae-mediated endocytosis. Since PhAsO and NEM reduced the endocytosis of ^{125}I-Tf almost completely, while

indomethacin had no effect, we conclude that TfR-ligand endocytosis is mediated via clathrin coated vesicles.

Modulation of the TfR was investigated not only by scavenging or extra addition of iron, but also by other modulators such as astrocytic factors. However, due to high non-specific binding it was not possible to perform experiments in a co-culture of BCEC and astrocytes (13), in which the BCEC are in direct contact with the astrocytes. Therefore, all experiments were performed in DMEM+ACM. This is important since it is known from literature that astrocytic factors induce and maintain BBB properties of the BCEC (1). In our experiments, removing astrocytic factors from the cell culture medium did not change the total TfR expression level, as this was 0.04 ± 0.01 and 0.05 ± 0.02 ng Tf/μg cell protein for DMEM+ACM and DMEM+S, respectively. Also for the extracellular TfR expression level and the association no differences were found in the presence or absence of ACM. The iron and Tf content in ACM were 3.3 and 7.3 μM, respectively, which is not different from 3.5 μM Fe and 5.2 μM Tf found for DMEM+S. Therefore, it can be concluded that astrocytes had not secreted extra iron or Tf. Furthermore, there appear to be no other astrocytic factors secreted in ACM, which influence the TfR expression and endocytosis.

To increase endocytosis of Tf by BCEC we also investigated the effect of the GTP-ase inhibitor T8, as it was shown in literature that T8 enhanced the transcytosis of Tf-conjugates through Caco-2 cells (20). 10 min pre-incubation with 0.25 mM T8 did not change Tf binding nor association. Increasing the concentration to 0.5 mM revealed the tendency to decrease the association of Tf. Therefore, we conclude from our experiments that T8 does not enhance endocytosis (as a first step of transcytosis) of Tf in BCEC. The difference between literature data (20) and our results may be explained by differences in cell type (endothelium vs epithelium), cellular metabolism (33), and species (bovine vs human).

The modulators that we have applied in the investigations described so far are either involved in the basic modulation of the TfR (iron), in the functional modulation of the BCEC (ACM) or the interference with intracellular processes (T8) and were tested at physiological conditions. In addition, we were also interested in the expression of the TfR under disease conditions, to investigate the possibility to target drugs to the BBB or brain under these conditions. Therefore, we have applied LPS, an inducer of inflammatory disease conditions and the free radical scavenger NAC, as an inhibitor of

inflammatory disease conditions. The effects of LPS on the binding and association after 24 h pre-incubation were small (figure 4 and 5). Following a 2 h pre-incubation the expression of the TfR was not changed (data not shown). However, due to the increase in the concentration of cellular protein (table II), the TfR expression per μg of cellular protein was decreased (figure 4A). Since shortly after inflammation, so-called acute phase proteins are formed (34), which can cause increased cellular protein levels, we have evaluated the effect of LPS on TfR expression, without correcting for this increase (figure 5).

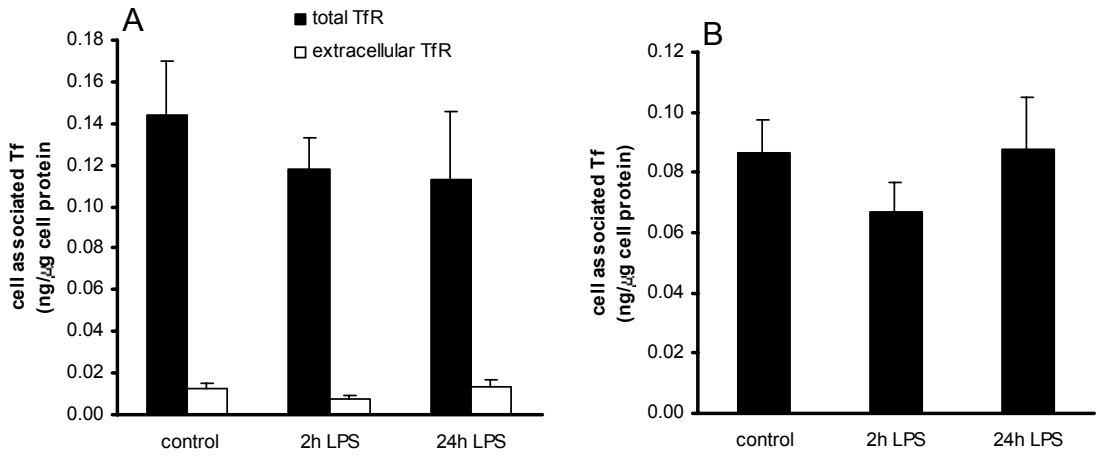


Figure 5: Total and extracellular binding (**A**) of ^{125}I-Tf to the TfR, determined at 4 °C, and endocytosis (**B**) of ^{125}I-Tf by the TfR, determined at 37 °C, after pre-incubation with 100 ng/ml LPS (2 or 24 h) and expressed in ng cell-associated Tf corrected for cellular protein levels of the control situation. Data represented are the specific binding, from at least 3 separate experiments performed in triplicate.

Therefore, we have corrected the TfR expression for the cellular protein of the control situation and not for the 2 h or 24 h LPS situation. In addition, the mRNA level of the TfR was also not changed after 2h pre-incubation with LPS (personal communication with Dr. PJ Gaillard), confirming that LPS has no effect on TfR expression level. Our results are consistent with the findings of Hallmann *et al* (35), who have studied the TfR in a murine brain derived endothelial cell line, cultured without astrocytic factors, in which they did not find an effect of LPS on Tf endocytosis. Furthermore, experiments conducted with cell lines of non-brain origin showed a decrease in TfR mRNA (36) or expression levels (21) due to LPS or oxidative stress.

Our results indicate that the TfR at the BBB is expressed equally under physiological conditions as well as inflammatory disease conditions.

The free radical scavenger NAC prevented disturbances of the BBB by LPS (26) and was also able to prevent downregulation of the TfR caused by oxidative stress in K562 cells (21). Therefore, we have investigated the effect of NAC on the expression of the TfR on BCEC. One hour or overnight pre-incubation with 10 mM NAC did not affect the TfR expression or association, nor did it affect the cellular protein levels (data not shown). In addition, the combination of NAC (1 h or overnight) and LPS (2 h) did not have an effect on the TfR expression levels nor endocytosis (figure 4). However, NAC was able to counteract the upregulation of cellular protein levels, caused by LPS (table II), resulting in "no change" in TfR expression per μg cellular protein compared to control.

In conclusion, we have characterised the TfR on BCEC, cultured in the presence of astrocytic factors. Although this receptor is present at the BBB, it is also present at other tissues. Our present research on primary cultured BCEC gives a extensive overview of the capacity and the use of the TfR at the BBB for drug delivery. The extracellular expression level of TfR is only 10 % of the total TfR expression, but the association of Tf is high. Furthermore, it was shown that 70 – 80 % of Tf is endocytosed by the TfR via clathrin-coated vesicles within 1 h. With DFO it was possible to increase the TfR expression level, as well as the association. This could have important implications for drug targeting to the brain via the TfR. In all other experimental conditions, including high iron concentrations, removal of ACM, inhibition of GTP-ase, and inflammatory conditions, the TfR expression level, as well as the association, was not changed. This shows that the TfR is present on the BBB in physiological as well as inflammatory disease conditions. Due to its constant expression the TfR is an interesting target for the selective delivery of (hydrophilic) drugs to the brain.

Acknowledgements

The authors thank Dr. Pieter Gaillard for his helpful discussion and technical comments, Dymphy Huntjens (MSc) for her input towards the NONMEM data analysis and Dr. Marijke Frohlich from the Central Clinical Laboratorium for iron and Tf measurements in ACM. This work was financially supported by STIGO (stimulation for innovative drug research, ZonMw) grant 014-80-003, the Hague, the Netherlands.

References

1. Rubin, L. L. and Staddon, J. M. (1999) The cell biology of the blood-brain barrier. *Annu Rev Neurosci* **22** 11-28
2. Pardridge, W. M. (1999) Blood-brain barrier biology and methodology. *J Neurovirol* **5** (6): 556-569
3. Pardridge, W. M. (1997) Drug delivery to the brain. *J Cereb Blood Flow Metab* **17** (7): 713-731
4. de Boer, A. G., van der Sandt, I. C. and Gaillard, P. J. (2003) The role of drug transporters at the blood-brain barrier. *Annu Rev Pharmacol Toxicol* **43** 629-656
5. Pardridge, W. (2001) Drug targeting, drug discovery, and brain drug development. In *Brain drug targeting - The future of brain drug development*, Press Syndicate of the University of Cambridge, Cambridge, pp. 1-12
6. Duffy, K. R. and Pardridge, W. M. (1987) Blood-brain barrier transcytosis of insulin in developing rabbits. *Brain Res* **420** (1): 32-38
7. Dehouck, B., Fenart, L., Dehouck, M. P., Pierce, A., Torpier, G. and Cecchelli, R. (1997) A new function for the LDL receptor: transcytosis of LDL across the blood-brain barrier. *J Cell Biol* **138** (4): 877-889
8. de Vries, H. E., Breedveld, B., Kuiper, J., de Boer, A. G., Van Berkel, T. J. and Breimer, D. D. (1995) High-density lipoprotein and cerebral endothelial cells in vitro: interactions and transport. *Biochem Pharmacol* **50** (2): 271-273
9. Shi, N., Zhang, Y., Zhu, C., Boado, R. J. and Pardridge, W. M. (2001) Brain-specific expression of an exogenous gene after i.v. administration. *Proc Natl Acad Sci U S A* **98** (22): 12754-12759
10. Moos, T. and Morgan, E. H. (2000) Transferrin and transferrin receptor function in brain barrier systems. *Cell Mol Neurobiol* **20** (1): 77-95
11. Baker, E. and Morgan, E. H. (1994) Iron Transport. In *Iron Metabolism in Health and Disease* (J. H. Brock, J. W. Halliday, M. Pippard and L. W. Powell, eds.), Saunders, London, pp. 63-95
12. Trinder, D. and Baker, E. (2003) Transferrin receptor 2: a new molecule in iron metabolism. *Int J Biochem Cell Biol* **35** (3): 292-296
13. Gaillard, P. J., Voorwinden, L. H., Nielsen, J. L., Ivanov, A., Atsumi, R., Engman, H., Ringbom, C., de Boer, A. G. and Breimer, D. D. (2001) Establishment and functional characterization of an in

vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur J Pharm Sci* **12** (3): 215-222

14. Nguyen, H. M., Cahill, C. M., McPherson, P. S. and Beaudet, A. (2002) Receptor-mediated internalization of [3H]-neurotensin in synaptosomal preparations from rat neostriatum. *Neuropharmacology* **42** (8): 1089-1098
15. Gumbleton, M., Abulrob, A. G. and Campbell, L. (2000) Caveolae: an alternative membrane transport compartment. *Pharm Res* **17** (9): 1035-1048
16. Casey, J. L., Koeller, D. M., Ramin, V. C., Klausner, R. D. and Harford, J. B. (1989) Iron regulation of transferrin receptor mRNA levels requires iron- responsive elements and a rapid turnover determinant in the 3' untranslated region of the mRNA. *Embo J* **8** (12): 3693-3699
17. Kuhn, L. C. (1991) mRNA-protein interactions regulate critical pathways in cellular iron metabolism. *Br J Haematol* **79** (1): 1-5
18. van Gelder, W., Huijskes-Heins, M. I., Cleton-Soeteman, M. I., van Dijk, J. P. and van Eijk, H. G. (1998) Iron uptake in blood-brain barrier endothelial cells cultured in iron- depleted and iron-enriched media. *J Neurochem* **71** (3): 1134-1140
19. Bridges, K. R. and Cudkowicz, A. (1984) Effect of iron chelators on the transferrin receptor in K562 cells. *J Biol Chem* **259** (21): 12970-12977
20. Xia, C. Q. and Shen, W. C. (2001) Tyrphostin-8 enhances transferrin receptor-mediated transcytosis in Caco-2- cells and increases hypoglycemic effect of orally administered insulin-transferrin conjugate in diabetic rats. *Pharm Res* **18** (2): 191-195
21. Malorni, W., Testa, U., Rainaldi, G., Tritarelli, E. and Peschle, C. (1998) Oxidative stress leads to a rapid alteration of transferrin receptor intravesicular trafficking. *Exp Cell Res* **241** (1): 102-116
22. Descamps, L., Dehouck, M. P., Torpier, G. and Cecchelli, R. (1996) Receptor-mediated transcytosis of transferrin through blood-brain barrier endothelial cells. *Am J Physiol* **270** (4 Pt 2): H1149-1158
23. Raub, T. J. and Newton, C. R. (1991) Recycling kinetics and transcytosis of transferrin in primary cultures of bovine brain microvessel endothelial cells. *J Cell Physiol* **149** (1): 141-151
24. Koenig, J. A., Edwardson, J. M. and Humphrey, P. P. (1997) Somatostatin receptors in Neuro2A neuroblastoma cells: ligand internalization. *Br J Pharmacol* **120** (1): 52-59
25. Rutledge, E. A., Mikoryak, C. A. and Draper, R. K. (1991) Turnover of the transferrin receptor is not influenced by removing most of the extracellular domain. *J Biol Chem* **266** (31): 21125-21130
26. Gaillard, P. J., de Boer, A. G. and Breimer, D. D. (2003) Pharmacological investigations on lipopolysaccharide-induced permeability changes in the blood-brain barrier in vitro. *Microvasc Res* **65** (1): 24-31
27. van Gelder, W., Huijskes-Heins, M. I., van Dijk, J. P., Cleton-Soeteman, M. I. and van Eijk, H. G. (1995) Quantification of different transferrin receptor pools in primary cultures of porcine blood-brain barrier endothelial cells. *J Neurochem* **64** (6): 2708-2715
28. Britton, R. S., Leicester, K. L. and Bacon, B. R. (2002) Iron toxicity and chelation therapy. *Int J Hematol* **76** (3): 219-228
29. Zhang, Y. and Pardridge, W. M. (2001) Rapid transferrin efflux from brain to blood across the blood-brain barrier. *J Neurochem* **76** (5): 1597-1600
30. Wheeler, M. B., Gelling, R. W., Hinke, S. A., Tu, B., Pederson, R. A., Lynn, F., Ehses, J. and McIntosh, C. H. (1999) Characterization of the carboxyl-terminal domain of the rat glucose-

dependent insulinotropic polypeptide (GIP) receptor. A role for serines 426 and 427 in regulating the rate of internalization. *J Biol Chem* **274** (35): 24593-24601

31. Pardridge, W. M., Eisenberg, J. and Yang, J. (1987) Human blood-brain barrier transferrin receptor. *Metabolism* **36** (9): 892-895
32. Wilcke, M. and Walum, E. (2000) Characterization of leptin intracellular trafficking. *Eur J Histochem* **44** (4): 325-334
33. de Boer, A. G., Gaillard, P. J. and Breimer, D. D. (1999) The transference of results between blood-brain barrier cell culture systems. *Eur J Pharm Sci* **8** (1): 1-4
34. Baumann, H. and Gauldie, J. (1994) The acute phase response. *Immunol Today* **15** (2): 74-80
35. Hallmann, R., Savigni, D. L., Morgan, E. H. and Baker, E. (2000) Characterization of iron uptake from transferrin by murine endothelial cells. *Endothelium* **7** (2): 135-147
36. Kim, S. and Ponka, P. (1999) Control of transferrin receptor expression via nitric oxide-mediated modulation of iron-regulatory protein 2. *J Biol Chem* **274** (46): 33035-33042

Chapter 4

Validation of the transferrin receptor for drug targeting to brain capillary endothelial cells *in vitro*

Journal of Drug Targeting (2004) 12 (3): 145 – 150

C.C. Visser, S. Stevanović, L.H. Voorwinden, P.J. Gaillard, D.J.A. Crommelin*, M. Danhof and A.G. de Boer

Leiden/Amsterdam Centre for Drug Research (LACDR), Leiden University, Division of Pharmacology, PO Box 9502, 2300 RA Leiden, The Netherlands

*Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), PO Box 80082, 3508 TB Utrecht, The Netherlands

Abstract

Recently, we have shown that transferrin (Tf) is actively endocytosed by the TfR on primary cultured bovine brain capillary endothelial cells (BCEC). The objective of this investigation is to determine whether the TfR can facilitate endocytosis of a (protein) model drug, using Tf as a targeting vector. Secondly, the mechanism of endocytosis was investigated. Horseradish peroxidase (HRP, 40 kDa) was chosen as a model drug, since it normally does not cross the BBB and its concentration in biological media can be easily quantified.

Tf-HRP conjugates (1:1) are actively and specifically endocytosed by BCEC *in vitro* in a concentration and time-dependent manner. At an applied concentration of 3 μg/ml, association (a combination of binding and endocytosis) of Tf-HRP reached equilibrium at a concentration of 2 ng/mg cell protein after 1 hour of incubation at 37 °C. This was approximately 3-fold higher compared to binding at 4 °C (0.6 ng/mg cell protein). Association of Tf-HRP was compared to BSA-HRP. After 2 h of incubation at 37 °C association levels were 5.2 and 2.5 ng/mg cell protein, for Tf-HRP and BSA-HRP, respectively. Under those conditions association of Tf-HRP could be inhibited to approximately 30 % of total association by an excess of non-conjugated Tf, but not with BSA, while association of BSA-HRP could be inhibited by both proteins. Furthermore, by using specific inhibitors of endocytotic processes, it was shown that association of Tf-HRP is via clathrin-coated vesicles. Association of Tf-HRP is inhibited by phenylarsine oxide (an inhibitor of clathrin-mediated endocytosis) to 0.4 ng/mg cell protein, but not by indomethacin, which inhibits formation of caveolae. Finally, following iron scavenging by deferoxamine mesylate (resulting in a higher TfR expression) a 5-fold increase in association of Tf-HRP to 15.8 ng/mg cell protein was observed.

In conclusion, the TfR is potentially suitable for targeting of a (protein) cargo to the BBB and to facilitate its endocytosis by the BCEC.

Introduction

The blood-brain barrier (BBB) plays an important role in maintaining homeostasis in the central nervous system. The brain capillary endothelial cells (BCEC) representing the BBB, differ from peripheral capillaries, in the sense that they are influenced by their surrounding neurons and astrocytes (1). As a result BCEC are characterised by narrow tight junctions, low pinocytotic activity and high metabolic activity, thereby, minimising both paracellular and transcellular transport of water soluble, high molecular weight compounds. Drug targeting to the brain often focuses on the utilisation of endogenous transporters at the BBB, such as the LDL-, HDL- or scavenger receptor (2-5). A more selective approach is utilisation of the transferrin receptor (TfR), expressed on BCEC. Mostly monoclonal antibodies to the TfR are used for brain drug targeting, but the endogenous ligand, transferrin (Tf) has also been used (6). From literature it is known that the TfR is highly expressed on BCEC and that its expression level is increased after scavenging iron from the cell culture medium (7). Recently, we have characterised, in a strictly quantitative manner, the expression of the TfR on primary cultured bovine BCEC in our *in vitro* co-culture model of the BBB. In these investigations it was shown that iron did modulate the TfR expression, but other modulators, such as astrocyte conditioned medium or lipopolysaccharide (LPS), had no effect on the TfR expression in bovine BCEC (8).

In the present study we investigated whether the TfR on BCEC can be used for selective delivery of a (protein) model compound. Tf is used as a targeting vector, and horseradish peroxidase (HRP) was chosen as a model compound, as it normally does not cross the BBB (9). Furthermore, HRP is readily measurable by colorimetric detection. Tf and HRP were conjugated in a 1:1 ratio, after which association (a combination of binding and endocytosis) of the Tf-HRP conjugate by BCEC was determined *in vitro*. Selectivity of the association was determined by addition of an excess of non-conjugated Tf, and by comparison to the association of BSA-HRP. Furthermore, the route of endocytosis was investigated by selective inhibition of either the clathrin-mediated pathway or the formation of caveolae (10-12). Finally, TfR expression was modulated using either the iron scavenger deferoxamine mesylate (DFO) or addition of extra iron ($FeCl_3$).

Experimental

Cell Culture

Primary brain capillary endothelial cells (BCEC) were cultured from isolated bovine brain capillaries as described before (13). Briefly, brain capillaries were seeded in type IV collagen and fibronectin-coated plastic culture flasks and cultured in a 1:1 mixture of DMEM (containing 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, non essential amino acids and 10% fetal calf serum) and astrocyte-conditioned medium (ACM), supplemented with 125 μg/ml heparin at 37 °C, 10% CO_2 for 4 - 5 days. ACM was obtained as described before (Gaillard *et al.* 2001). At 70 % confluence the BCEC were passaged with trypsin-EDTA and seeded into a type IV collagen coated 96 wells plate at a density of 10,000 cells/well. Subsequently, BCEC were cultured in the same medium at 37 °C, 10% CO_2 for 4 - 5 days.

Preparation of Tf-HRP and BSA-HRP conjugates

Bovine Tf and BSA were conjugated to HRP according to the protocol of Alpha Diagnostics Int., in which pre-activated HRP is conjugated to a primary amine of the protein to be conjugated. Briefly, 1 mg Tf or BSA was dissolved in conjugation buffer and added to 1 mg of pre-activated HRP. After overnight incubation at 4 °C, blocking buffer was added and the mixture was further incubated for 1 h. After removal of non-conjugated HRP by centrifugation over a 50 kDa membrane, the conjugates were stored at 4°C.

The conjugates were examined by polyacrylamide gel electrophoresis under reducing conditions, followed by protein staining (Coomassie). Staining occurred at around 120 kDa (Tf-HRP) or 100 kDa (BSA-HRP), indicating that most of the protein was conjugated. Bands around 40 kDa (HRP), 80 kDa (Tf) or 60 kDa (BSA) were hardly visible, indicating that the presence of free protein was negligible.

Association- and binding studies

BCEC were checked under the microscope for confluency and morphology (13). 1 hour *prior* to the experiment the medium was changed to serum-free DMEM to deplete the cells from endogenous Tf. BCEC were incubated with 0.5 – 12 μg/ml Tf-HRP for 2 h (concentration range), or with 3 μg/ml Tf-HRP for 15 – 240 min (time range) in 100 μl PBS at 37 °C, to determine association, which is a combination of binding and uptake, or at 4 °C, to determine binding only. Non-specific binding was determined in the presence of 1.5 mg/ml of non-conjugated Tf when using 3 μg/ml Tf-HRP or with 0.25 – 6 mg/ml for the concentration range. The specificity of the association was determined by incubation of BCEC for 2 h at 37 °C with 3 μg/ml Tf-HRP or BSA-HRP in the absence or presence of 1.5 mg/ml non-conjugated Tf or BSA.

After incubation, BCEC were washed 6 times with 200 µl ice-cold PBS and solubilised for at least 2 h at room temperature with 40 µl Na-deoxycholic acid (0.1 % in MilliQ) for determination of HRP activity (adapted from (14)) or cellular protein.

Endocytosis experiments

To determine which endocytotic processes were involved in the association of Tf-HRP, BCEC were pre-incubated for 10 min with PhAsO (10 µM), or indomethacin (50 µg/ml). Concentrations and pre-incubation time were modified from Gumbleton *et al.* (10) and Nguyen *et al.* (12). Subsequently, BCEC were incubated with 3 µg/ml Tf-HRP in 100 µl PBS in the presence of the inhibitors at 37 °C for 1 h. Again 1.5 mg/ml Tf was used to determine non-specific binding. BCEC were washed and solubilised as described above.

TfR modulation by iron

BCEC were pre-incubated for 24 h with 1 mM DFO or $FeCl_3$ before incubation with Tf-HRP (3 µg/ml in the absence or presence of 1.5 mg/ml Tf, for 2 h). Cells were washed and solubilised as described above.

HRP activity assay and protein assay

HRP activity was determined using a colorimetric assay. Briefly, cell lysates were incubated with TMB liquid substrate for 20 min, after which 0.5 M H_2SO_4 was added to stop the reaction. HRP (0 – 2 ng/ml) was used for a standard curve and absorption was read at 450 nm.

Cellular protein contents were determined using Biorad DC reagents and BSA for a standard curve (0 – 400 µg/ml). Absorption was read at 690 nm.

Data analysis

All data are represented as specific binding and are means of at least 3 individual experiments, performed in quadruplicate. All data are represented as mean ± SD. The time curve was analysed according to an one-phase exponential association, in which $A = A_{max} * (1 - e^{-k*x})$. In this formula A is the association, A_{max} the maximal association, x is the time and k is the association constant. GraphPad Prism version 3.00 (GraphPad Software, San Diego, California, USA) was used for this analysis, while statistical analysis was performed by one way ANOVA, followed by a Bonferroni multiple comparison test, using GraphPad InStat version 3.00 (GraphPad Software, San Diego, California, USA).

Materials

Culture flasks were obtained from Greiner (Alphen a/d Rijn, the Netherlands) and 96 wells plates from Corning Costar (Cambridge, MA, USA). PBS, DMEM, supplements and fetal calf serum were purchased from BioWhittaker Europe (Verviers, Belgium). Type IV collagen, heparin, trypsin-EDTA, endothelial cell trypsin, NEM, PhAsO, indomethacin, DFO, $FeCl_3 \cdot 6\ H_2O$, HRP, sodium deoxycholic acid and TMB liquid substrate were obtained from Sigma (Zwijndrecht, the Netherlands) and fibronectin from Boehringer Mannheim (Almere, the Netherlands). The HRP conjugation kit was purchased from Alpha Diagnostics Int. (San Antonio, TX, USA), bovine holo-Tf and BSA were obtained from ICN Pharmaceuticals (Zoetermeer, the Netherlands) and Bio-Rad DC protein assay reagents from Bio-Rad Laboratories (Veenendaal, the Netherlands).

Results

Association- and binding studies

Incubation of BCEC with 0.5 – 10 μg/ml Tf-HRP at 37 °C showed a concentration-dependent, specific association (a combination of binding and endocytosis), which was linear up to 10 μg/ml (figure 1A). Binding at 4 °C was also concentration dependent, but considerably less (figure 1A). These findings indicate that there was active endocytosis of Tf-HRP at 37 °C.

The time dependent association experiments (figure 1B) showed that saturation of the TfR was reached after approximately 60 minutes. Maximal association was 2.13 ± 0.12 ng Tf-HRP/mg cell protein and the association constant k was estimated at 0.051 ± 0.014 min^{-1}. Binding at 4 °C was approximately 4 times lower than association at 60, 120 and 180 min, indicating that association at 37 °C is a mixture of binding and endocytosis.

For both the concentration and the time dependent experiments, non-specific association was approximately 40 % of total association, which was independent of the applied concentration of the conjugate. For the determination of non-specific association a 500-fold excess of non-conjugated Tf was used. Increasing this concentration did not decrease the non-specific association further (data not shown). Figure 2 shows that the association at an applied concentration of 3 μg/ml Tf-HRP could be displaced with Tf, but not with BSA. This indicates that association is largely a

TfR mediated process. In addition, association of BSA-HRP can be displaced by both BSA and Tf, suggesting non-specific association.

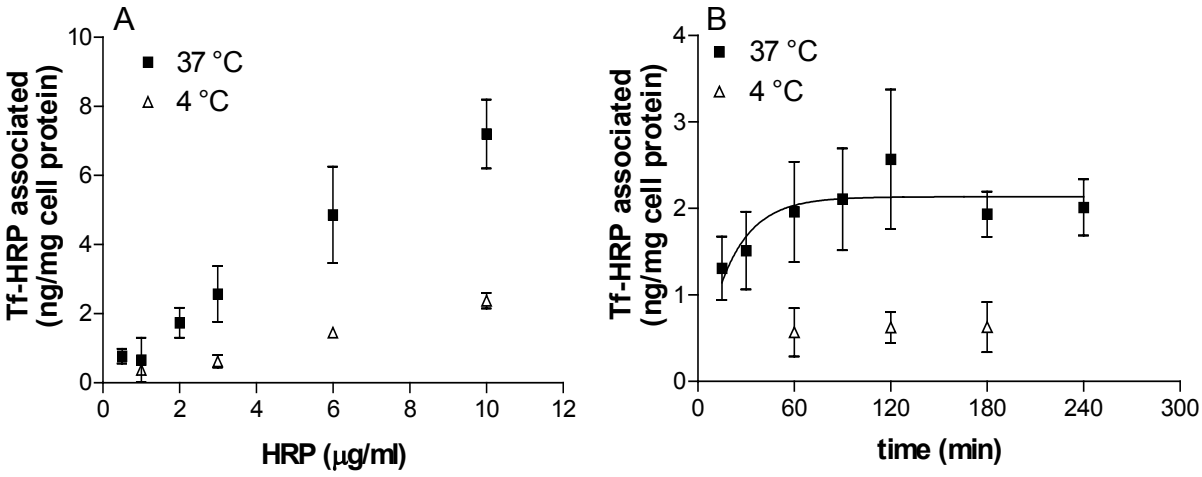


Figure 1: Concentration (**A**) and time (**B**) dependent association and binding of Tf-HRP. Incubation of BCEC with Tf-HRP (0.5 – 10 μg/ml) for 2 h at 37 °C or at 4 °C showed a concentration dependent association of Tf-HRP, which is linear up to 10 μg/ml. The association at 37 °C was approximately 4-fold higher at each concentration tested. Incubation of BCEC with 3 μg/ml Tf-HRP for 15 – 240 minutes at 37 °C or at 4 °C, showed that after approximately 60 minutes equilibrium was reached for the association (determined at 37 °C). Binding was around 4-fold lower at 60, 120 and 180 min. Data represented (mean ± SD; n = 3 experiments, each performed in quadruplicate) are the specific association (total association corrected for non-specific association); non-specific association was approximately 40% of total association (independent of the concentration).

Endocytosis experiments

BCEC were pre-incubated with PhAsO, or indomethacin for 10 min, followed by an one hour incubation with 3 μg/ml Tf-HRP at 37 °C in the presence of the inhibitors. Figure 3 shows that PhAsO inhibited the uptake of Tf-HRP almost completely while indomethacin inhibited the endocytosis by 50 %, suggesting that endocytosis occurred mainly via a clathrin-mediated pathway. Binding studies at 4 °C showed that these inhibitors had no effect on the binding of Tf-HRP (data not shown).

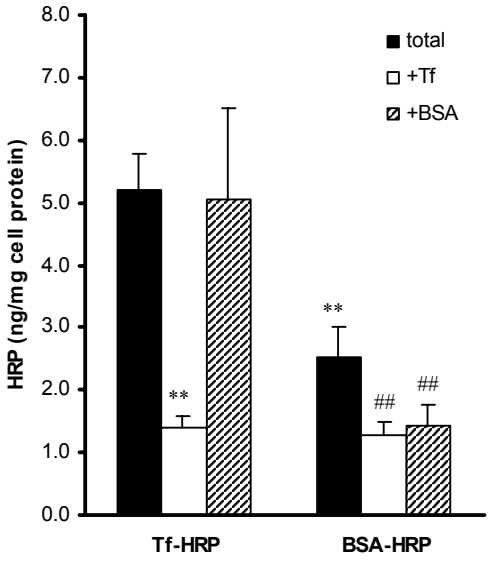


Figure 2: Association of Tf-HRP (3 μg/ml, 2 h at 37 °C) was reduced by a 500-fold excess of non-conjugated Tf, but not by an equal concentration of BSA. Total association of BSA-HRP (3 μg/ml, 2 h at 37 °C) was approximately 50 % of the association of Tf-HRP, and was inhibited by both Tf and BSA. Data represented are mean ± SD (n = 3 experiments, each performed in quadruplicate), ** $P < 0.01$, Tf-HRP +Tf, or total BSA-HRP vs total Tf-HRP, ## $P < 0.01$, BSA-HRP +Tf or BSA-HRP +BSA vs total BSA-HRP, Bonferroni multiple comparisons test.

TfR modulation by iron

After 24 h pre-incubation with the iron scavenger DFO at 37 °C, association of Tf-HRP was increased approximately 2.5-fold from 3.4 ± 1.2 ng/mg cell protein to 8.5 ± 1.1 ng/mg cell protein (figure 4), reflecting upregulation of the TfR. After pre-incubation with $FeCl_3$ the association was not significantly changed (figure 4).

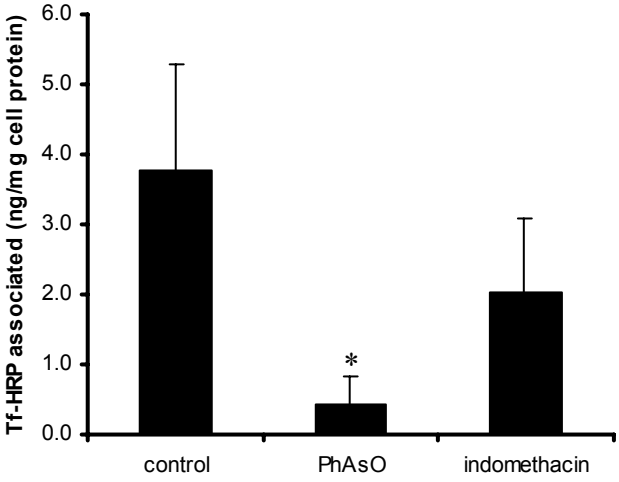


Figure 3: BCEC were incubated with 3 μg/ml Tf-HRP for 1 h. The specific association of Tf-HRP was inhibited by PhAsO (1 μM), but not by indomethacin (50 μg/ml), indicating that endocytosis of Tf-HRP occurs via clathrin-coated vesicles. Data represented are mean ± SD (n = 4 experiments, each performed in quadruplicate), * $P < 0.05$, PhAsO vs control, Bonferroni multiple comparisons test.

Discussion

The TfR expressed at the BBB is an interesting target for drug delivery to the brain (15, 16). Recently, we have characterised, in a strictly quantitative manner, the TfR expression and endocytosis on our BCEC *in vitro*. The TfR expression and endocytosis was found to be dependent on the presence of iron in the cell culture medium, but independent of other modulators such as astrocytic factors or LPS (8). These studies were performed with ^{125}I-labelled Tf, which constitutes only a small modification to Tf. Therefore, in the present research the potential of the TfR to facilitate endocytosis of a larger compound conjugated to Tf was investigated. As a model compound HRP was chosen, as it normally does not cross the BBB (9) and is easily measurable via colorimetric detection. Our studies demonstrate that Tf-HRP is taken up by BCEC *in vitro* via a clathrin-mediated process.

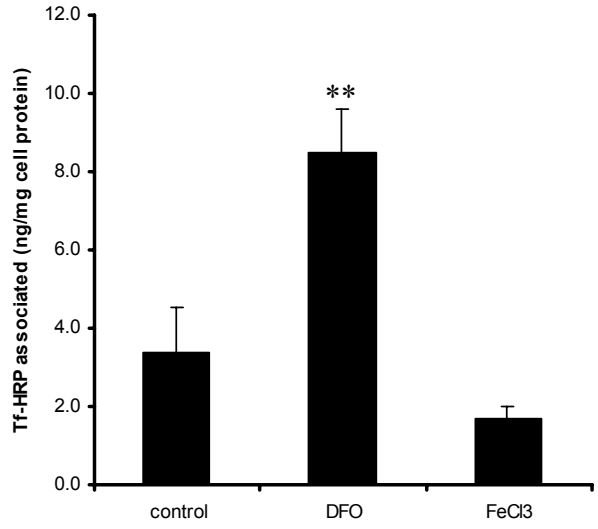


Figure 4: BCEC were incubated with 3 μg/ml Tf-HRP for 2 h at 37 °C, after 24 h pre-incubation with DFO or $FeCl_3$ (both 1 mM). DFO caused a 2.5-fold increase in the specific association of Tf-HRP while $FeCl_3$ had no significant effect. Data represented are mean ± SD (n = 3 experiments, each performed in quadruplicate), ** $P < 0.01$, control vs DFO, Bonferroni multiple comparison test.

The association of Tf-HRP at 37 °C, which is a combination of specific binding and endocytosis, increased linearly up to 10 μg/ml Tf-HRP added (figure 1A), and reached equilibrium after 60 min incubation (figure 1B). The binding of Tf-HRP (determined at 4 °C) was approximately 4-fold lower than the association at 37 °C, indicating that endocytosis took place via an active process. Figure 2 shows that the uptake of Tf-HRP was inhibited by 1.5 mg/ml non-conjugated Tf, but not by BSA, confirming that Tf-HRP was selectively endocytosed via the TfR. The non-specific association of Tf-HRP

was approximately 40 % and remained constant at all concentration and time points. This is higher than the non-specific association we have found for ^{125}I-Tf, which was 15 – 20 % (8). However, in the present experiments increasing the concentration of non-conjugated Tf up to 6 mg/ml did not decrease the association of Tf-HRP further (data not shown). The association of BSA-HRP was inhibited by 1.5 mg/ml BSA (figure 2), as well as by Tf. This indicates that association of BSA-HRP occurred by a non-specific process, probably adsorptive mediated endocytosis (4, 17).

By gel electrophoresis it was shown that the conjugate also contained minor quantities of free Tf and free HRP, which can interfere with the association of Tf-HRP. However, the presence of small quantities of free Tf does not significantly interfere with our results, as a 500-fold excess of non-conjugated Tf was still necessary to displace binding and endocytosis of the Tf-HRP conjugate. In theory however, the free HRP might have interfered with our results, since it can be taken up by BCEC via non-specific processes or by Tf-induced endocytotic vesicles. Therefore, a control experiment was performed in which BCEC were incubated with HRP (3 μg/ml) and Tf (3 μg/ml or 1.5 mg/ml). In this experiment Tf concentrations were either similar to the HRP concentration (mimicking the total binding/association) or to the level of non-specific binding (i.e. a 500-fold excess *versus* the Tf-HRP concentration used for non-specific binding). Association of free HRP was 0.55 ng/mg cell protein at 37 °C and 0.47 ng/mg cell protein at 4 °C. Addition of both the high and the low concentration of Tf decreased both the association and the binding of HRP to approximately 0.40 ng/mg cell protein. Since the association of free HRP was at the same level as the non-specific association of the Tf-HRP conjugate and was not increased by Tf, we conclude that the free HRP in the Tf-HRP conjugate did not interfere with the results we obtained.

Association of Tf-HRP could be inhibited by PhAsO, but to a lesser and non-significant degree by indomethacin (figure 3), indicating that endocytosis of the conjugate occurs primarily via a clathrin-mediated pathway. In these experiments incubation with the Tf-HRP conjugate and inhibitors was only 1 h, while for all other experiments 2 h was chosen. Increasing the incubation time led to a loss of protein, while an incubation time of less than 1 h could not be used, since the association between receptor and ligand did not reach equilibrium in this time (figure 1B). The binding of the Tf-HRP conjugate, determined at 4 °C, was not altered by these inhibitors (data not shown), indicating that only the active processes are influenced by PhAsO and

indomethacin. In general, receptor mediated uptake is associated with clathrin coated vesicles (10). The present results are generally similar to those obtained with ^{125}I-labelled Tf (8), indicating that both the native ligand, as well as the conjugated ligand, are endocytosed by BCEC via clathrin-mediated endocytosis by the TfR.

Pre-incubation with DFO (figure 4), resulted in an approximately 2.5-fold increase in association of Tf-HRP (from 3.4 ± 1.2 ng/mg cell protein to 8.5 ± 1.1 ng/mg cell protein). This was due to the increased TfR expression level, as well as the increased endocytosis (8). These data suggest that Tf conjugated to HRP is endocytosed by BCEC *in vitro* in a manner similar to native Tf. Pre-incubation with $FeCl_3$ had no significant effect on the association of the conjugate, although endocytosis seems to be lower.

From literature it is already known that Tf or OX-26, an antibody against the rat TfR can be used for drug targeting to the brain (15, 18), although the mechanism of endocytosis is still not fully understood. Drug targeting to the TfR *in vivo* is mainly done with OX26, since Tf encounters too much competition from endogenous Tf. However, for the characterisation of the TfR on bovine BCEC *in vitro* only polyclonal antibodies against the TfR exist, which are not specific enough. Therefore, we have chosen the endogenous ligand Tf for the validation of the TfR for drug targeting. Our previous and present data show that native Tf (^{125}I-labelled) and Tf conjugated to HRP are both endocytosed via clathrin-coated vesicles. The ability of Tf to facilitate the endocytosis of a 40 kDa protein by BCEC has major implications for the treatment of diseases of the BBB, such as meningitis, but also for diseases in the brain. Pardridge *et al* (16) have shown *in vivo* effects of neurotrophins, targeted to the TfR, in brain ischemia in a rat model. Furthermore, Shi *et al* (19) have shown that a peptide nucleic acid conjugate, also targeted to the TfR, could be used for antisense imaging of gene expression in the brain *in vivo*. The experiments by Pardridge (16) and Shi *et al* (19) show that the conjugates are stable enough to target to an endosomal route, associated with the TfR. Further research to evaluate the use of the TfR for brain drug delivery in human will be necessary. An important issue in this respect is the selection of a suitable targeting vector. Tf itself is not a logical choice since competition from endogenous Tf may be a problem. Furthermore, for long term use of an antibody against the TfR, a humanized antibody needs to be developed, to prevent immunogenicity. A small ligand, that binds specifically to the TfR on brain capillary endothelium is an attractive approach to overcome these problems.

In conclusion, our data show that the TfR is suitable for targeting and delivery of a compound (protein) to BCEC and possibly to the brain. Furthermore, scavenging of iron causes an increase in TfR expression and endocytosis, and is thereby increasing the delivery of drugs via the TfR.

Acknowledgements

The authors thank Arjen Brink (MSc) for the preparation of the Tf-HRP conjugates.
This work was financially supported by grant 014-80-003 from STIGO (stimulation for innovative drug research, ZonMw), the Hague, the Netherlands and grant 10F02.17 from the Hersenstichting (Brain Foundation), the Hague, the Netherlands.

References

1. Rubin, L. and Staddon, J. (1999) The cell biology of the blood-brain barrier. *Annu. Rev. Neurosci.* **22** 11-28
2. de Vries, H. E., Kuiper, J., de Boer, A. G., van Berkel, T. J. and Breimer, D. D. (1993) Characterization of the scavenger receptor on bovine cerebral endothelial cells in vitro. *J Neurochem* **61** (5): 1813-1821
3. de Vries, H. E., Breedveld, B., Kuiper, J., de Boer, A. G., Van Berkel, T. J. and Breimer, D. D. (1995) High-density lipoprotein and cerebral endothelial cells in vitro: interactions and transport. *Biochem Pharmacol* **50** (2): 271-273
4. De Boer, A. G., Van Der Sandt, I. C. and Gaillard, P. J. (2003) The role of drug transporters at the blood-brain barrier. *Annu Rev Pharmacol Toxicol* **43** 629-656
5. Dehouck, B., Fenart, L., Dehouck, M. P., Pierce, A., Torpier, G. and Cecchelli, R. (1997) A new function for the LDL receptor: transcytosis of LDL across the blood-brain barrier. *J Cell Biol* **138** (4): 877-889
6. Pardridge, W. M. (1998) CNS drug design based on principles of blood-brain barrier transport. *J Neurochem* **70** (5): 1781-1792
7. Moos, T. and Morgan, E. H. (2000) Transferrin and transferrin receptor function in brain barrier systems. *Cell Mol Neurobiol* **20** (1): 77-95
8. Visser, C. C., Voorwinden, L. H., Crommelin, D. J., Danhof, M. and de Boer, A. G. (2004) Characterization and modulation of the transferrin receptor on brain capillary endothelial cells. *Pharm Res* **21** (5): 761-769

9. Banks, W. A. and Broadwell, R. D. (1994) Blood to brain and brain to blood passage of native horseradish peroxidase, wheat germ agglutinin, and albumin: pharmacokinetic and morphological assessments. *J Neurochem* **62** (6): 2404-2419
10. Gumbleton, M., Abulrob, A. G. and Campbell, L. (2000) Caveolae: an alternative membrane transport compartment. *Pharm Res* **17** (9): 1035-1048
11. Wilcke, M. and Walum, E. (2000) Characterization of leptin intracellular trafficking. *Eur J Histochem* **44** (4): 325-334
12. Nguyen, H. M., Cahill, C. M., McPherson, P. S. and Beaudet, A. (2002) Receptor-mediated internalization of [3H]-neurotensin in synaptosomal preparations from rat neostriatum. *Neuropharmacology* **42** (8): 1089-1098
13. Gaillard, P. J., Voorwinden, L. H., Nielsen, J. L., Ivanov, A., Atsumi, R., Engman, H., Ringbom, C., de Boer, A. G. and Breimer, D. D. (2001) Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur J Pharm Sci* **12** (3): 215-222
14. Fukushima, H., Iwasaki, M., Yosie, O. and Igarashi, H. (2001) Induction of reduced endothelial permeability to horseradish peroxidase by factor(s) of human astrocytes and bladder carcinoma cells: detection in multi-well plate culture. *Methods Cell Sci* **23** (4): 211-219
15. Li, H. and Qian, Z. M. (2002) Transferrin/transferrin receptor-mediated drug delivery. *Med Res Rev* **22** (3): 225-250
16. Pardridge, W. M. (2002) Blood-brain barrier drug targeting enables neuroprotection in brain ischemia following delayed intravenous administration of neurotrophins. *Adv Exp Med Biol* **513** 397-430
17. Bickel, U., Yoshikawa, T. and Pardridge, W. M. (2001) Delivery of peptides and proteins through the blood-brain barrier. *Adv Drug Deliv Rev* **46** (1-3): 247-279
18. Pardridge, W. M. (2002) Drug and gene targeting to the brain with molecular Trojan horses. *Nat Rev Drug Discov* **1** (2): 131-139
19. Shi, N., Boado, R. J. and Pardridge, W. M. (2000) Antisense imaging of gene expression in the brain in vivo. *Proc Natl Acad Sci U S A* **97** (26): 14709-14714

Section 3

Liposomes tagged with transferrin for drug delivery

Chapter 5

Coupling of metal containing homing devices to liposomes via a maleimide linker: use of TCEP to stabilise thiol-groups without scavenging metals

Journal of Drug Targeting (2004), in press

C.C. Visser, L.H. Voorwinden, L.R. Harders, M. Eloualid, L. van Bloois[*], D.J.A. Crommelin[*], M. Danhof and A.G. de Boer

Leiden/Amsterdam Centre for Drug Research (LACDR), Leiden University, Division of Pharmacology, PO Box 9502, 2300 RA Leiden, The Netherlands

[*]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), PO Box 80082, 3508 TB Utrecht, The Netherlands

Abstract

Liposomes for drug delivery are often prepared with maleimide groups on the distal end of PEG to enable coupling of homing devices, such as antibodies, or other proteins. EDTA is used to stabilise the thiol group in the homing device for attachment to the maleimide. However, when using a homing device that contains a metal, EDTA inactivates this by scavenging of the metal. Holo-transferrin (Tf) containing 2 iron atoms (Fe^{3+}), has a much higher affinity for the transferrin receptor than apo-Tf (which does not contain any Fe^{3+}). To couple Tf to a liposome, the introduction of a thiol group is necessary. During this process, by using N-succinimidyl S-acetylthioacetate (SATA), followed by 2 - 3 hr coupling to the liposomes, Fe^{3+} is scavenged by EDTA. This causes a decreased affinity of Tf for its receptor, resulting in a decreased targeting efficiency of the liposomes.

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) is a sulfhydryl reductant that is often used in protein biochemistry. We found that TCEP (0.01 mM) does not scavenge Fe^{3+} from Tf and is able to protect thiol groups for the coupling to maleimide. Furthermore, TCEP does not interfere with the maleimide coupling itself.

In this communication we describe the preparation of liposomes, focussing on the coupling of Tf to the maleimide linker at the distal end of PEG, without loosing Fe^{3+} from Tf. This method can be applied to other metal-containing homing devices as well.

Introduction

Drug delivery to the brain is very difficult due to the presence of the blood-brain barrier (BBB). Zhang *et al* (2003) have shown that liposomes targeted to the transferrin receptor (TfR) at the BBB can enhance the delivery of plasmid DNA (1). However, they used an antibody against the rat TfR as a homing device. We are interested in the mechanism of endocytosis via the TfR on bovine brain capillary endothelial cells *in vitro*. For the bovine TfR only polyclonal antibodies exist, which are not specific. Therefore, our goal was to develop a drug delivery system using the endogenous ligand for this receptor, transferrin (Tf), as a homing device. Tf is a 80 kDa protein folded into 2 identical subunits, which each can carry one iron (Fe^{3+}) atom. Holo-Tf contains 2 iron atoms and has a high affinity for the TfR at physiological pH, while apo-Tf does not contain any iron and has at least a 1000-fold lower affinity for the TfR (2).

For the preparation of liposomes we have adapted the method used by Heeremans *et al* (1992) and Mastrobattista *et al* (1999) who have used maleimide to couple a homing device to liposomes (3, 4). Certain homing devices such as Fab-fragments contain a thiol group for coupling to maleimide. Tf does not expose a free thiol group. However, by using N-succinimidyl S-acetylthioacetate (SATA) it can easily be introduced to a primary amine in an one-step reaction. It is important that the thiol group is protected from oxidation. This can be achieved by avoiding contact with oxygen (working under constant N_2) and/or by addition of EDTA (1 mM) to all aqueous solvents that are used. However, EDTA has the capacity to scavenge iron from Tf, thereby, decreasing its affinity for the TfR. Other SH-protectants often have a thiol group themselves (e.g. β-mercaptoethanol, dithiothreitol (DTT)), that competes for maleimide coupling. In contrast, tris(2-carboxyethyl)phosphine (TCEP, figure 1), that is often used in protein biochemistry, does not contain a thiol group. Furthermore, TCEP has proven to be a good reductant (5). Therefore, we have applied TCEP for the preparation of Tf-tagged liposomes.

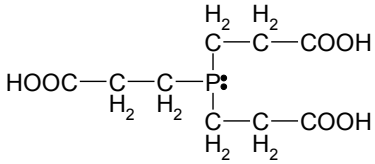


Figure 1: Structure of Tris(2-carboxyethyl)phosphine (TCEP)

For this communication, we have determined the amount of thiol groups introduced in Tf, as well as their stability. Furthermore, we determined whether Tf retained its iron during the preparation of Tf-tagged liposomes. Finally, the coupling efficiency of Tf to liposomes containing maleimide was determined in the presence of EDTA and TCEP.

Experimental

Transferrin

Tf was modified with a thiol group using SATA (6). Tf and SATA (1:8 molar ratio) were incubated in HBS pH 7.0 for 1 h at room temperature under constant shaking (step 1, figure 2). Free SATA was removed by centrifugation over 30 kDa cut-off filters. Directly before coupling to maleimide the thiol group was activated by incubation with 0.1 M hydroxylamine for 45 min at room temperature (step 2, figure 2). The amount and stability of the thiol groups was determined with Ellman's reagent (7). The presence of EDTA or TCEP did not affect the Ellman's determination of thiol groups. The iron determination was based on the inspection of UV-VIS absorption spectra at 280 and 460 nm (8). Holo-Tf (10 mg/ml) was incubated for 24 h at room temperature with EDTA (1 mM) or TCEP (0.1 or 0.01 mM). As a control apo-Tf (10 mg/ml) was loaded with $FeCl_3$ and sodium citrate (9). $FeCl_3$ and sodium citrate were mixed in a 1:10 ratio, before addition of 2 mol Fe^{3+} per mol Tf.

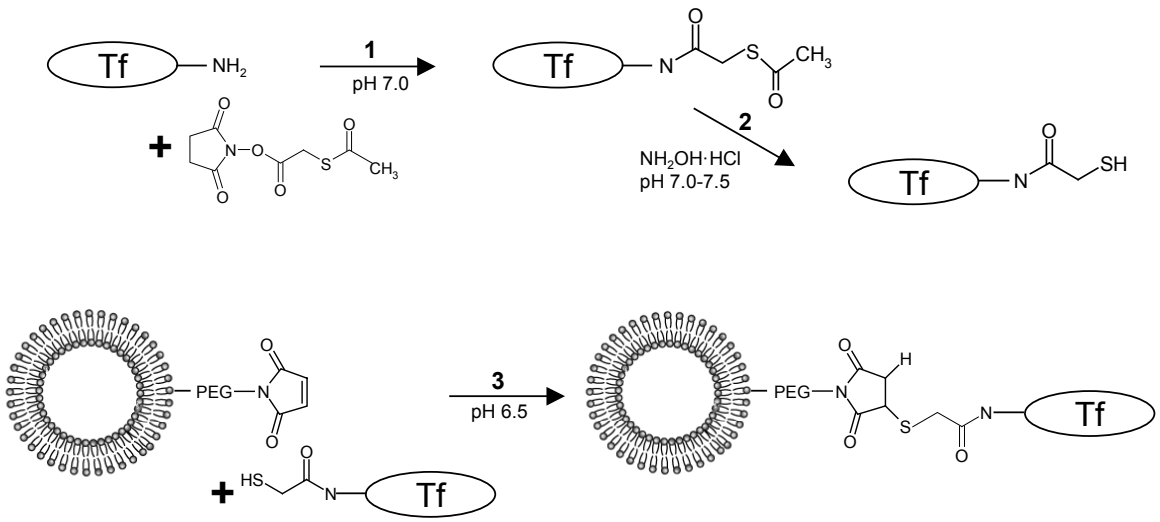


Figure 2: Reaction scheme of the thiolation of Tf with N-succinimidyl-S-acetylthioacetate (SATA, step 1), the activation of thiolated Tf with hydroxylamine HCl (step 2) and the conjugation of Tf to liposomes, bearing maleimide-PEG-DSPE (step 3).

Liposome preparation

Liposomes were prepared according to Heeremans *et al* (1992) and Mastrobattista *et al* (1999) with some modifications (3, 4). Briefly, liposomes were prepared of EPC-35 and cholesterol in a 2:1 molar ratio, with 2.5 % PEG_{2000}-DSPE and 2.5 % PEG_{2000}-maleimide-DSPE. Briefly, after evaporation of organic solvents the lipid film was resuspended in HBS pH 6.5 and liposomes were extruded 8 times through 200 & 100 nm polycarbonate filters using a hand-extruder from Avanti Polar Lipids (Alabaster, AL, USA). Directly after extrusion liposomes (8 – 12 mM phospholipid (PL)) were incubated with SATA modified Tf at a final amount of 1 – 4 mg Tf per 9 μmol PL in the presence of EDTA (1 mM) or TCEP (0.01 mM) for 2 h at room temperature under constant shaking (step 3, figure 2). The incubation volume was 1 – 2 ml, depending on the concentrations PL and Tf. After incubation with Tf, the liposomes were incubated with L-cystein for 30 min, to block all excess maleimide groups. N-ethylmaleimide (NEM, 8 mM) was added to the liposomes to stop the coupling reaction before separation of free Tf and liposomes by ultracentrifugation (60.000 g, 30 min, 10 °C). After ultracentrifugation liposomes were resuspended in HBS pH 7.0 without any additives.

Characterisation of liposomes

After preparation, the phospholipid content was determined according to Rouser (10), and the protein content was determined with the Biorad protein assay. Furthermore, size and polydispersity were determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd. Malvern, UK).

Materials

Bovine holo- and apo-transferrin, $FeCl_3$ and hydroxylamine-HCl were purchased from ICN Pharmaceuticals (Zoetermeer, the Netherlands). SATA, EDTA, TCEP, HEPES, L-cystein and cholesterol were obtained from Sigma (Zwijndrecht, the Netherlands), sodium citrate from J.T. Baker (Deventer, the Netherlands) sodium chloride from Merck (Amsterdam, the Netherlands). The Biorad protein assay was obtained from Bio-Rad Laboratories (Veenendaal, the Netherlands) and DiD from Molecular Probes (Leiden, the Netherlands). EPC-35 was purchased from Lipoid GmbH (Ludwigshafen, Germany), PEG_{2000}-DSPE from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and PEG_{2000}-maleimide-DSPE from Shear Water Corporation (Huntsville, AL, USA). Vivaspin columns are obtained from Vivascience AG (Hannover, Germany) and polycarbonate filters for extrusion from Nuclepore (Pleasanton, USA).

Results

Transferrin

Tf:SATA ratios of 1:4, 1:8, 1:12 and 12:1 were used (data not shown), but introduction of thiol groups was most effective at a Tf:SATA ratio of 1:8. After de-protection with hydroxylamine in the presence of EDTA 2.2 mol SH per mol Tf was obtained after 1 h incubation (table I). Using TCEP in a similar concentration (1 mM) 5.6 mol SH per mol Tf was detected after 1 h. This number dropped slightly over 24 h. With 0.1 and 0.01 mM TCEP 2.5 and 2.3 mol SH per mol Tf was detected after 1 h incubation, respectively. After 24 h at room temperature the amount of SH groups in the presence of EDTA was decreased with 50%, while with all concentrations of TCEP this decrease was less. Moreover, when heating Tf for 2 h at 37 °C TCEP proved to be a better stabiliser for thiol groups than EDTA (table I). Without hydroxylamine present, TCEP (0.1 and 0.01 mM) were able to de-protect the thiol group in SATA-modified Tf: after 1 h incubation 1.3 mol SH per mol Tf was detected.

Table I: Tf was modified with SATA (1:8 molar ratio) and subsequently incubated for 1 h with hydroxylamine to de-protect the thiol group in the presence or absence of EDTA or TCEP. The amount of SH groups per Tf molecule were determined directly after de-protection (1 h), 24 hours after de-protection (24h), or after 24 hours at room temperature, followed by 2 hours at 37 °C (24 h + 2 h 37 °C).

SATA-modified Tf in the presence of	SH/Tf (mol/mol)		
	1 h	24 h	24 h + 2 h 37 °C
no stabiliser	2.3	1.3	0.7
EDTA (1 mM)	2.2	1.1	0.6
TCEP (1 mM)	5.6	4.9	3.9
TCEP (0.1 mM)	2.5	1.9	1.6
TCEP (0.01 mM)	2.3	1.6	1.4
Tf (not-modified)	0.2	0.3	0.3

From UV-VIS analysis it was shown that both holo- and apo-Tf showed an absorption maximum at 280 nm (typical for proteins) but only holo-Tf showed a second absorption maximum at 460 nm (figure 3). This latter maximum is an indication for the presence of Fe^{3+}, although this was only visible at concentrations higher than 5 mg/ml. Incubation of holo-Tf with EDTA showed a small decrease in the iron peak already after 2 hour (data not shown), after 24 h this decrease was approximately 50 % (figure 3). The absorption spectra of Tf in the absence or presence of TCEP (0.1 or 0.01 mM) showed no difference (figure 3). Apo-Tf, incubated for 24 h with $FeCl_3$, had a similar absorption spectrum compared to holo-Tf. $FeCl_3$ and sodium citrate without Tf present did not show an absorption peak at 460 –470 nm (data not shown).

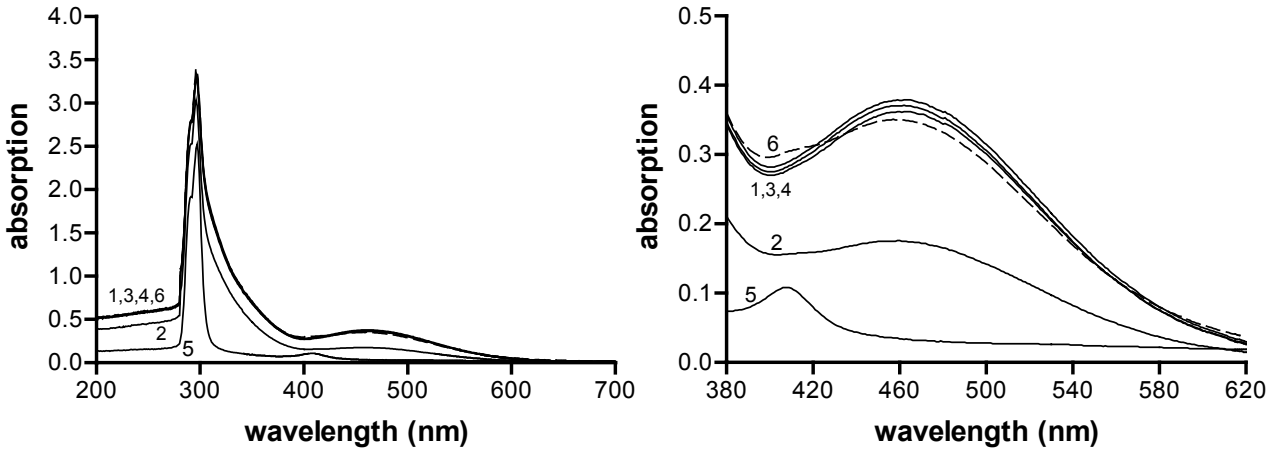


Figure 3: UV-VIS spectra of holo-Tf (10 mg/ml) in the absence (1) or presence of EDTA (1 mM, 2) or TCEP (0.1 or 0.01 mM, lines 3 and 4, respectively) and apo-Tf (10 mg/ml) in the absence (5) or presence (6) of $FeCl_3$ and sodium citrate. A typical protein peak is visible around 280 nm (left panel), while only for holo-Tf and apo-Tf incubated for 24 h with Fe^{3+} a peak around 460 nm is visible (right panel). EDTA is able to scavenge Fe^{3+}, as is shown by the decrease in absorption at 460 nm, TCEP does not scavenge Fe^{3+} after 24 h incubation (right panel).

Liposomes

Coupling of SATA modified-Tf to liposomes containing maleimide groups reached a maximum at 0.33 mg/μmol PL (figure 4). As expected, no difference in the extent of

coupling was obtained when this was performed either in the presence of EDTA or TCEP.

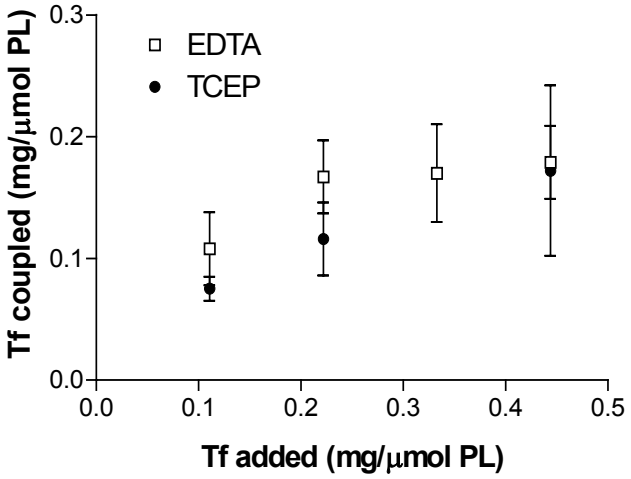


Figure 4: The amount of coupled Tf per μmol phospholipid (PL) is plotted against the added amount of Tf per μmol PL. This shows that the coupling of Tf is saturable at amounts higher than 0.25 mg Tf per μmol PL. The amount of coupling is not changed in the presence of TCEP (0.01 mM) compared to EDTA (1 mM).

Discussion

Targeting drugs to the brain is often aimed at the TfR on the BBB (1, 11). Recently, we have characterised the TfR on BBB endothelial cells (12). Furthermore, we have validated the use of the TfR for targeting drugs to the BBB by using Tf conjugated to horseradish peroxidase (13). By applying liposomes, it is possible to increase the ratio of target site delivered drug molecules per Tf homing device molecule. This is particularly true for small drug molecules. Therefore, we are interested in the preparation of Tf-tagged liposomes. During the preparation of Tf-tagged liposomes, it is important that Tf retains its iron, since holo-Tf (diferric Tf) has at least a 1000-fold higher affinity for the TfR than mono-ferric or apo-Tf (*2*). Each molecule Tf can bind two iron atoms with simultaneous binding of CO_3^{2-} (14). At physiological pH the binding of iron and the anion is strong, therefore, we do not expect that during the preparation of liposomes the binding of iron and the anion is changed. However, metal scavengers that can reach the iron binding site of Tf might affect the binding of iron to Tf.

Initially, we used EDTA during the preparation of the liposomes as a stabilising agent for the introduced thiol groups (6), but EDTA also has metal-scavenging properties. Alternatively, TCEP (figure 1) is a new thiol stabilising agent which does not

have the capacity to scavenge iron from Tf, as is shown in the UV-VIS spectra in figure 3. Holo-Tf has 2 peaks, one around 280 nm, which is typical for proteins, and a second peak between 460 and 470 nm. The latter is specific for iron, and is decreased to approximately 50 % after 24 h in the presence of EDTA (1 mM). This process is time dependent (data not shown), indicating that Tf is loosing its iron. In the presence of TCEP (0.1 and 0.01 mM) Tf retains its iron, since the peak at 460-470 nm does not change during incubation for 24 h.

The amount of thiol groups introduced after the SATA reaction and de-protection with hydroxylamine, is similar in the presence of EDTA (1 mM) or TCEP (0.1 and 0.01 mM) or "no stabiliser" (table I, 1h). After 24 h at room temperature and after 24 h at room temperature in combination with 2 h at 37 °C, more thiol groups per mol Tf were found in the presence of TCEP (0.1 and 0.01 mM) than in the presence of EDTA (table I). This indicates that TCEP is a better stabiliser of thiol groups. Kratz *et al* (1998) have prepared Tf conjugates of doxorubicin in the presence of EDTA (1 mM) (15). However, the EDTA was only present during the insertion of a thiol group with iminothiolane (1 hour incubation) and not during the coupling to maleimide. Our method required a longer incubation in the presence of EDTA, which caused a dissociation of iron from Tf. Therefore, we have used TCEP (0.1 mM) for the preparation of Tf-tagged liposomes.

A higher concentration TCEP (1 mM) caused a strong increase in the amount of thiol groups (5.6 mol SH per mol Tf after 1 h), indicating that Tf might be modified, and was probably not suitable for targeting anymore. Therefore, we have decided to use concentrations of 0.1 or 0.01 mM TCEP. When no hydroxylamine was present TCEP (0.1 and 0.01 mM) alone could also activate thiol groups in Tf (1.3 mol SH per mol Tf), although this occurred less efficiently. These experiments suggest that, although TCEP is a known reductant for disulphides (5, 16), it can also cleave a thioester bond.

Coupling of Tf to maleimide on the distal end of PEG-liposomes in the presence of EDTA (1 mM) of TCEP (0.1 mM) was concentration dependent, as is shown in figure 4. The coupling in the presence of TCEP tended to be slightly lower, although this difference was not significant. Highest coupling efficiencies were obtained at the lower concentrations, where more than 75 % of the added Tf was coupled, while at Tf concentrations higher than 3 mg/ 9 μmol PL coupling efficiency was 40 – 50 %. Burmeister Getz et al (5) have shown that TCEP (0.1 mM) inhibited the labelling of

myosin (a protein with a free thiol group) with a fluorescent label, containing a maleimide group, substantially. They also found that increasing the TCEP concentration led to a decrease in fluorescent label. However, they have used a ratio of maleimide to thiol of 3:1, while we have used a 4.4 – 17.5 fold excess of maleimide during the coupling of Tf to liposomes. Other reductants such as β-mercaptoethanol or DTT are very effective in their protection of free thiol groups, but they possess a thiol group themselves. Therefore, it is not possible to use β-mercaptoethanol or DTT for maleimide coupling. All liposomes were similar in size (170 – 200 nm) and had a polydispersity index lower than 0.25, indicating that after coupling of Tf in the presence of EDTA or TCEP we obtained similarly sized liposomes.

The objective of our research is to explore the use of the TfR for drug delivery to the brain. For this we initially used horseradish peroxidase (HRP) conjugated to a primary amine of Tf, which was specifically endocytosed by the TfR (13). To extent our research we want to apply liposomes loaded with HRP and tagged with Tf. For the preparation of these liposomes Tf is modified at a primary amine, similar as for the preparation of Tf-HRP conjugates. Therefore, we expect that the binding to and endocytosis by the TfR of Tf-tagged liposomes is not altered due to the SATA reaction and subsequent binding to maleimide-PEG-PE on the liposomal surface.

In conclusion, we have successfully applied the use of TCEP for the coupling of homing devices containing metal ions to maleimide groups on liposomes. In addition, for an efficient coupling it is important to protect the thiol groups in the homing device from oxidation. We have shown for our ligand Tf that TCEP has the advantage of stabilising thiol groups without scavenging iron from Tf.

References

1. Zhang, Y., Calon, F., Zhu, C., Boado, R. J. and Pardridge, W. M. (2003) Intravenous nonviral gene therapy causes normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental parkinsonism. *Hum Gene Ther* **14** (1): 1-12
2. Baker, E. and Morgan, E. (1994) Iron Transport. In *Iron Metabolism in Health and Disease* (J. Brock, J. Halliday, M. Pippard and L. Powell, eds.), Saunders, London, pp. 63-95

3. Heeremans, J. L., Kraaijenga, J. J., Los, P., Kluft, C. and Crommelin, D. J. (1992) Development of a procedure for coupling the homing device glu-plasminogen to liposomes. *Biochim Biophys Acta* **1117** (3): 258-264
4. Mastrobattista, E., Storm, G., van Bloois, L., Reszka, R., Bloemen, P. G., Crommelin, D. J. and Henricks, P. A. (1999) Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells. *Biochim Biophys Acta* **1419** (2): 353-363
5. Burmeister Getz, E., Xiao, M., Chakrabarty, T., Cooke, R. and Selvin, P. R. (1999) A comparison between the sulfhydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. *Anal Biochem* **273** (1): 73-80
6. Bloemen, P. G., Henricks, P. A., van Bloois, L., van den Tweel, M. C., Bloem, A. C., Nijkamp, F. P., Crommelin, D. J. and Storm, G. (1995) Adhesion molecules: a new target for immunoliposome-mediated drug delivery. *FEBS Lett* **357** (2): 140-144
7. Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch Biochem Biophys* **82** (1): 70-77
8. Aasa, R., Malmstroem, B. G. and Saltman, P. (1963) The Specific Binding of Iron(Iii) and Copper(Ii) to Transferrin and Conalbumin. *Biochim Biophys Acta* **75** 203-222
9. Holbein, B. E. (1981) Enhancement of Neisseria meningitidis infection in mice by addition of iron bound to transferrin. *Infect Immun* **34** (1): 120-125
10. Rouser, G., Fkeischer, S. and Yamamoto, A. (1970) Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5** (5): 494-496
11. Bickel, U., Yoshikawa, T. and Pardridge, W. M. (2001) Delivery of peptides and proteins through the blood-brain barrier. *Adv Drug Deliv Rev* **46** (1-3): 247-279
12. Visser, C. C., Voorwinden, L. H., Crommelin, D. J., Danhof, M. and de Boer, A. G. (2004) Characterization and modulation of the transferrin receptor on brain capillary endothelial cells. *Pharm Res* **21** (5): 761-769
13. Visser, C. C., Stevanović, S., Heleen Voorwinden, L., Gaillard, P. J., Crommelin, D. J., Danhof, M. and De Boer, A. G. (2004) Validation of the Transferrin Receptor for Drug Targeting to Brain Capillary Endothelial Cells In Vitro. *J Drug Target* **12** (3): 145-150
14. Morgan, E. H. (1996) Iron metabolism and transport. In *Hepatology. A textbook of liver disease*, Vol. 1 (D. Zakin and T. D. Boyer, eds.), Saunders, Philadelphia, pp. 526-554
15. Kratz F., Beyer U., Roth T., Tarasova N., Collery P., Lechenault F., Cazabat A., Schumacher P., Unger C., Falken U., 1998. Transferrin conjugates of doxorubicin: synthesis, characterization, cellular uptake, and in vitro efficacy. J Pharm Sci 87, 338-346
16. Krijt, J., Vackova, M. and Kozich, V. (2001) Measurement of homocysteine and other aminothiols in plasma: advantages of using tris(2-carboxyethyl)phosphine as reductant compared with tri-n-butylphosphine. *Clin Chem* **47** (10): 1821-1828

Chapter 6

Targeting liposomes with protein drugs to the blood-brain barrier *in vitro*

Submitted for publication

C.C. Visser, S. Stevanović, L.H. Voorwinden, L. van Bloois[*], P.J. Gaillard, M. Danhof, D.J.A. Crommelin[*], and A.G. de Boer

Leiden/Amsterdam Centre for Drug Research (LACDR), Leiden University, Division of Pharmacology, PO Box 9502, 2300 RA Leiden, The Netherlands

[*]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), PO Box 80082, 3508 TB Utrecht, The Netherlands

Abstract

In this study, we aim to target pegylated liposomes loaded with HRP and tagged with Tf to the BBB *in vitro*. Liposomes were prepared with the post-insertion technique. Tf was measured indirectly by measuring iron via atomic absorption spectroscopy. All liposomes were around 100 nm in diameter, contained 5 - 13 μg HRP per μmol phospholipid and 63 – 74 Tf molecules per liposome (lipo Tf) or no Tf (lipo C).

Brain capillary endothelial cells (BCEC) were incubated with liposomes at 4 °C (to determine binding) or at 37 °C (to determine association, i.e. binding + endocytosis) and the HRP activity, rather than the HRP amount was determined in cell lysates. Association of lipo Tf was 2 – 3 fold higher than association of lipo C. Surprisingly, the binding of lipo Tf at 4 °C was 4 fold higher than the association of at 37 °C. This is explained by intracellular degradation of endocytosed HRP.

In conclusion, successful targeting of liposomes loaded with protein or peptide drugs to BCEC may be hampered by intracellular degradation of the drug. However, it may also be an advantage to target the liposomal content to the lysosomes (e.g. in the treatment of lysosomal storage disease). Our experiments suggest that liposomes release some of their content within the BBB, making targeting of liposomes to the TfR on BCEC an attractive approach for brain drug delivery.

Introduction

The central nervous system (CNS) is protected by the blood-brain barrier (BBB). This barrier is located at the interface between blood and brain and its primary function is to maintain homeostasis in the brain. Unique features, such as tight junctions, low vesicular transport and high metabolic activity accomplish this barrier function (1). Drug delivery to the brain is limited due to the BBB. Only small molecules (molecular weight lower than 600 Da) can pass the BBB paracellularly or transcellularly, depending on their lipophilicity (2). However, high molecular weight drugs, such as (recombinant) proteins, peptides or DNA, do not cross the BBB (3).

Over the years, many drug targeting and delivery strategies have been explored. Drug delivery strategies to the brain involve chimeric proteins, in which the protein drug is covalently linked to a transport vector (4). For the delivery of antisense drugs, encapsulation of the antisense in targeted liposomal drug carriers has been applied (4, 5). In our previous work we have shown that drug targeting to the transferrin receptor (TfR) with conjugates of transferrin (Tf) and horseradish peroxidase (HRP) shows accumulation of Tf-HRP in brain capillary endothelial cells *in vitro* (6).

In this study, we extend our investigations to deliver proteins to the BBB. Therefore, we have incorporated HRP in Tf-tagged pegylated liposomes. By using liposomes the amount of drug molecules per targeting vector (i.e. Tf) can be increased. Furthermore, it is not necessary to chemically modify the drug molecule, and the drug molecule is protected from degradation in serum. Also, for targeting drugs to the TfR at the BBB, liposomes have an additional advantage, since it is possible to circumvent the efflux transporter P-glycoprotein (7). HRP was chosen as a model compound, since it does not cross the BBB (8, 9) and is readily quantified. Liposomes were prepared according to the post-insertion technique (10, 11). Tf was tagged to the distal end of the polyethylene glycol chain (PEG) via a maleimide-thiol coupling. Non-tagged (lipo C) and Tf-tagged (lipo Tf) liposomes were prepared, containing 5 - 13 μg HRP per μmol phospholipid. Lipo Tf contained 63 – 74 Tf molecules per liposome.

Primary brain capillary endothelial cells (BCEC) were incubated with liposomes at 4 ºC (to determine binding) or at 37 ºC (to determine association, i.e. a combination of binding and endocytosis). Incubation was performed based on adding equal HRP concentrations in liposomal form, to be able to compare non-tagged liposomes with Tf-

tagged liposomes. From these studies with Tf-tagged liposomes and from our previous research on Tf-HRP conjugates (6), we are able to distinguish between route and rate of uptake of Tf-tagged liposomes and Tf-protein conjugates, as well as intracellular trafficking and fate of HRP.

Experimental

Cell culture

Primary brain capillary endothelial cells (BCEC) were cultured from isolated bovine brain capillaries as described before (12). Briefly, brain capillaries were seeded in type IV collagen and fibronectin-coated plastic culture flasks and cultured in a 1:1 mixture of DMEM (containing 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, non essential amino acids and 10% fetal calf serum) and astrocyte-conditioned medium (ACM), supplemented with 125 μg/ml heparin at 37 °C, 10% CO_2 for 4 - 5 days. ACM was obtained as described before (12). ACM is added to the cell culture medium to induce BBB properties of the BCEC, while the TfR is not influenced by addition of ACM (13). At 70 % confluence the BCEC were passaged with trypsin-EDTA and seeded into a type IV collagen coated 96 wells plate at a density of 15,000 cells/well. Subsequently, BCEC were cultured for 4 – 5 days at 37 °C, 10% CO_2.

Preparation of liposomes containing HRP, using the post-insertion technique

Tf was modified with a thiol group using SATA (1:8 molar ratio) as described before (14). Micelles of PEG_{2000}-DSPE and PEG_{2000}-maleimide-DSPE (in a molar ratio of 1:1) were prepared by hydration of the lipid film in 0.25 ml HEPES buffered saline (HBS), pH 6.5. Directly after hydration micelles were incubated with SATA modified Tf (1 or 3 mg Tf per 9 μmol phospholipid) in the presence of TCEP (0.01 mM) for 2 h at room temperature under constant shaking. To block all excess maleimide groups, micelles were incubated with L-cystein for 30 min, subsequently 1 mM NEM was added to block free thiol groups. Free maleimide groups need to be blocked to prevent non-specific reactions with other components during the preparation or the use of liposomes.

Liposomes were prepared of EPC-35 and cholesterol in a 2:1 molar ratio, by resuspending a lipid film in HBS with a final HRP concentration of 300 μg/μmol phospholipid (PL). Liposomes (approximately 10 mM PL) were extruded stepwise, through 200, 100, 80 and 50 nm polycarbonate filters (4 times per filter size) using a hand-extruder from Avanti Polar Lipids (Alabaster, AL, USA). External HRP was removed by ultracentrifugation (60,000 g, 45 min, 10 °C).

Liposomes and PEG micelles were mixed for 2 h at 40 °C to obtain liposomes with 5% total PEG. This method was modified from earlier published protocols (10, 11). After incubation, the mixture was separated on a Sepharose CL4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). The fractions containing the liposomes were concentrated by ultracentrifugation (60,000 g, 30 min, 10 °C). After ultracentrifugation liposomes were resuspended in HBS pH 7.0 and stored at 4 °C. The liposomes were used for experiments within 3 weeks, during which the HRP activity did not change.

Association of liposomes by BCEC

BCEC were checked under the microscope for confluency and morphology (12). BCEC were incubated with liposomes, based on the HRP concentration. BCEC were incubated in 50 µl DMEM with 10 % serum (DMEM+S) with 1 – 12 µg/ml liposomes for 2 h (concentration range), or with 3 µg/ml liposomes for 1 – 8 hour (time range). Incubation was performed at 37 °C, to determine association, which is a combination of binding and uptake, or at 4 °C, to determine binding only.

After incubation, BCEC were washed 6 times with 200 µl ice-cold PBS and solubilised for at least 2 h at room temperature with 40 µl Na-deoxycholic acid (0.1 % in MilliQ) for determination of HRP activity (adapted from (15)) or cellular protein. All data are represented as mean ± SD (performed in quadruplicate). Statistical analysis was performed by an unpaired t-test, using GraphPad InStat version 3.00 (GraphPad Software, San Diego, California, USA).

Analytical Methods

The PL content was determined according to Rouser (16). Size and polydispersity index (p.i.) were determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd. Malvern, UK). The p.i. is a measure of the particle size distribution and can range form 0 (monodisperse) to 1 (polydisperse).

HRP activity was determined using a colorimetric assay. Briefly, cell lysates or liposomes were incubated with TMB liquid substrate for 20 min, after which 0.5 M H_2SO_4 was added to stop the reaction. HRP (0 – 2 ng/ml) was used for a standard curve and absorption was read at 450 nm. HRP activity in 0.1 % Na-deoxycholic acid was not significantly different from samples in MilliQ. Cellular protein contents were determined using Biorad DC reagents and BSA for a standard curve (0 – 400 µg/ml). Absorption was read at 690 nm.

The Tf concentration was determined indirectly, by measuring the iron content with atomic absorption spectroscopy (Perkin Elmer 3100 atomic absorption spectrometer, Boston, MA, USA). Iron was measured at 248.3 nm against a standard curve (0 – 400 µg/ml Fe), samples were prepared in water. The calculation of the Tf concentration is based on the assumption that each Tf contains 2 iron atoms.

The number of Tf molecules per liposome is calculated with

$$(E \cdot 4\pi r^2 \cdot number\ of\ monolayers) / (MW \cdot (PL \cdot SH_{EPC} + CHOL \cdot SH_{CHOL})),$$

in which E is the g protein per mol total lipid, r is the radius of one liposome in nm, the number of monolayers for a 100 nm liposome is 2, MW is the molecular weight of Tf (77,000), PL is the phospholipid in mol, CHOL is the cholesterol in mol and SH is the surface area of EPC-35 and cholesterol headgroups. The surface area of EPC-35 and cholesterol is 0.70 nm^2 and 0.28 nm^2, respectively (17). For this calculation we assume that EPC-35 and cholesterol are spread evenly over the liposomal bilayer and are incorporated in a 2 : 1 molar ratio (17). For HRP the same formula is used, but with a MW of 40,000.

Materials

Culture flasks were obtained from Greiner (Alphen a/d Rijn, the Netherlands) and 96 wells plates from Corning Costar (Cambridge, MA, USA). PBS, DMEM, supplements and fetal calf serum were purchased from BioWhittaker Europe (Verviers, Belgium). Type IV collagen, heparin, trypsin-EDTA, endothelial cell trypsin, HRP, sodium deoxycholic acid, TMB liquid substrate, SATA, TCEP, HEPES, L-cystein and cholesterol were obtained from Sigma (Zwijndrecht, the Netherlands) and fibronectin from Boehringer Mannheim (Almere, the Netherlands). Bovine holo-Tf, BSA and hydroxylamine-HCl were obtained from ICN Pharmaceuticals (Zoetermeer, the Netherlands) and sodium chloride from Merck (Amsterdam, the Netherlands). EPC-35 was purchased from Lipoid GmbH (Ludwigshafen, Germany), PEG_{2000}-DSPE from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and PEG_{2000}-maleimide-DSPE from Shear Water Corporation (Huntsville, AL, USA). Vivaspin columns are obtained from Vivascience AG (Hannover, Germany) and polycarbonate filters for extrusion from Nuclepore (Pleasanton, USA). The Biorad protein assay was obtained from Bio-Rad Laboratories (Veenendaal, the Netherlands).

Results and Discussion

Characterisation of liposomes

Liposomes were prepared according to the post-insertion technique. Liposome batch 1 was prepared with PEG-micelles incubated with 1 mg Tf, while PEG-micelles for batch 2 and 3 were incubated with 3 mg Tf. Table I summarises the PL, HRP and Tf concentrations. All liposomes were around 100 nm and have polydispersion index (p.i.) values lower than 0.2, indicating that the liposomes have a rather narrow size

distribution. Liposomes were used within 3 weeks for experiments, during that time the increase in size and p.d. was less than 10 %. PL and HRP concentrations differed between batches 1, 2 and 3. Batch 1 had a different HRP concentration for lipo C (11.8 μg/μmol PL) and lipo Tf (6.4 μg/μmol PL). The encapsulation efficiency calculated from the final HRP concentration (table I) and the added amount of 300 μg HRP per μmol PL was 1.5 – 4.6 %. Corvo *et al* (18) have shown that for liposomes containing superoxide dismutase the encapsulation efficiency decreased upon extrusion. However, extrusion is necessary to obtain liposomes of approximately 100 nm. When calculating the average number of HRP molecules per liposome, based on the HRP activity assay rather than the amount of HRP, only 10 - 26 HRP molecules per liposome were incorporated. This low incorporation might be due to the extrusion as well as due to loss of HRP activity: we did not determine the amount of HRP by a protein determination due to interference by Tf, but have used the activity of the incorporated HRP for these calculations.

The average number of Tf molecules exposed from the outer surface was 63 – 74 per liposome. Based on previous experiments we expected an approximate 2-fold increase in Tf-tagging when a 3-fold higher amount of Tf was added to liposomes (14). But, the difference in the number of Tf molecules per liposome between batch 1 (1 mg Tf) or batch 2 and 3 (3 mg Tf) was relatively small. Therefore, we have not been able to determine the influence of the number of Tf molecules at the liposome surface on the association of liposomes by BCEC. Although we have not been able to determine the incorporation efficiency of the PEG micelles into the liposomes, this small difference in Tf-tagging between liposome batches may be the result of the post-insertion method that is used for the preparation of HRP containing liposomes.

Tf containing two iron atoms has the highest affinity for the TfR. Recently, we have described the preparation of Tf-tagged liposomes without loosing Fe from Tf, for which we determined the Tf concentration by a protein assay (14). However, the liposomes used for these experiments contain HRP, which interferes with the protein determination of Tf. Therefore, the determination of the iron concentration has been used to indirectly measure the Tf concentration. This method was verified using empty Tf-tagged liposomes in which Tf was determined using a protein assay as well as using AAS to determine Fe (data not shown).

Table I: Characterisation of liposomes, based on their phospholipid (PL) content, size, polydispersion index (p.i., a p.i. < 0.2 indicates a rather narrow particle size distribution), HRP concentration and the number of Tf molecules per liposome. Each batch contained non-tagged liposomes (lipo C) and Tf-tagged liposomes (lipo Tf).

		PL (mM)	Size (nm)	p.i.	HRP/PL (µg/µmol)	Tf molecules per liposome
batch 1	*lipo C*	9.8	110	0.15	11.8	
	lipo Tf	10.8	112	0.09	6.4	63
batch 2	*lipo C*	9.5	81	0.20	4.5	
	lipo Tf	8.9	98	0.16	5.7	70
batch 3	*lipo C*	5.0	101	0.09	13.7	
	lipo Tf	4.8	113	0.16	11.1	74

Comparing Tf-HRP conjugates (1:1 ratio) and Tf-tagged liposomes containing HRP, there is no increase in the amount of drug (HRP) per targeting vector (Tf), when using liposomes for protein drugs. However, Vingerhoeds *et al* (19) and Mastrobattista *et al* (20) have shown that liposomes with diphtheria toxin chain A (MW 21,000) are effective at the target site. Therefore, incorporation of a few potent protein drug molecules may be efficient if the drug is potent enough at low concentrations.

Binding and association of liposomes by BCEC

BCEC were incubated with liposomes. The chosen concentrations were based on equal HRP concentrations for comparison of non-tagged and Tf-tagged liposomes. We ascertained that the amount of liposomes (based on their PL concentration) that was added to the cells did not affect the cells at the concentrations used (data not shown).

After 2 hours of incubation of BCEC with liposomes at 37 °C, the difference in association of lipo C and lipo Tf was 2 – 4 fold (figure 1 A). This was found consistently in all experiments and was independent of the batch of liposomes used. Extensive concentration and time studies at 37 °C with all batches of liposomes were performed. For this, BCEC were either incubated with 1 – 12 μg/ml HRP, for 2 hours or with 3 μg/ml HRP for 1 – 8 hours. In two experiments the association of lipo Tf seemed saturated after 4 hours of incubation, but this was not reproducible. In general, the association of lipo Tf by BCEC increased both depending on time and concentration (data not shown), while association of lipo C was less dependent on time and concentration. This indicates that Tf-tagged liposomes are associated by BCEC via a different mechanism than non-tagged liposomes. We found that the difference in association between lipo C and lipo Tf was highest after 2 hours of incubation with liposomes containing 3 μg/ml HRP (figure 1A).

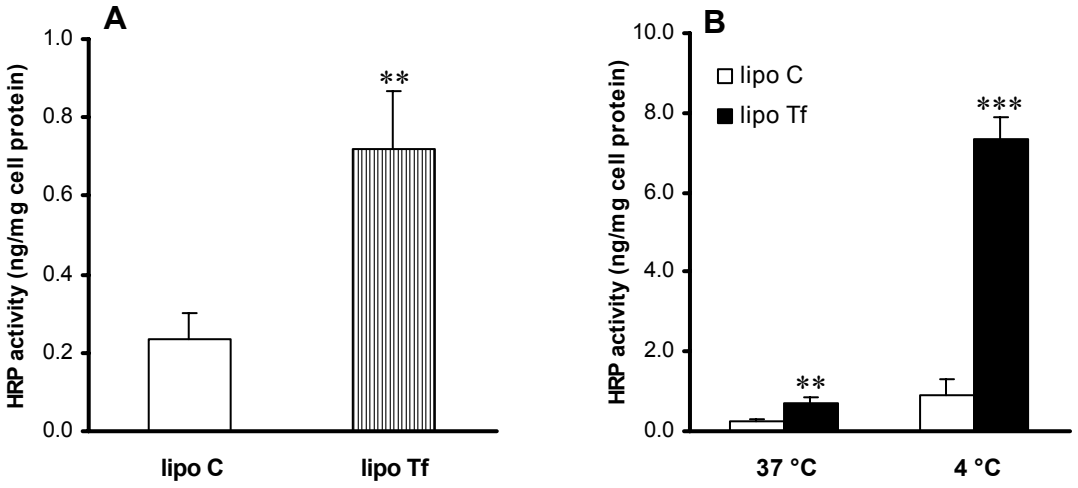


Figure 1: BCEC were incubated with liposomes (3 μg/ml HRP) for 2 hours at 37 ºC (to determine association) or at 4 ºC (to determine binding). Figure **1A** shows the association of liposomes, in figure **1B** the association is compared to the binding of liposomes. Tf-tagged liposomes show higher binding than association, please notice the difference in Y-axis. These graphs are representative for all data generated during these experiments. ** $P < 0.01$, *** $P < 0.001$, unpaired t-test.

Binding studies at 4 °C (figure 1B) with liposomes (3 μg/ml HRP) show that lipo Tf displayed a 4 – 8 -fold higher binding to BCEC than lipo C. All experiments were performed in the presence of serum and thus in the presence of endogenous Tf. Tf is, in fact, a high abundance protein in serum (21). The concentration Tf in serum is between 1.7 and 2.7 g/L (22). In our cell culture and in the binding and association studies we used 10 % serum in the incubation medium. Previously, we have reported that DMEM with 10 % serum contained 5.2 μM Tf, which is approximately 400 μg/ml (13). The liposome solution contains 290 – 500 μg/ml Tf, which is approximately 20 times further diluted in the experiments, indicating that liposomal Tf is lower than serum Tf. Therefore, the TfR might already be saturated with Tf. We have performed experiments with liposomes in the presence of serum to mimic *in vivo* conditions and to minimise the association of non-tagged liposomes. BCEC under physiological conditions show a very low, non-specific vesicular uptake of solutes from the blood (1). However, we found that when the experiments were performed in DMEM without serum or in PBS, non-tagged liposomes display a high association (data not shown). When incubating BCEC with liposomes in the presence of serum, the association of non-tagged liposomes was low.

The binding at 4 °C of lipo Tf (data not shown) was not time dependent, while the association (37 °C) was. The experiments with Tf-HRP showed saturation of both binding and association by BCEC after 1 hour incubation (6). This is consistent with the binding of Tf-tagged liposomes, which did not change between 1 and 8 hours. The association at 37 °C of Tf-tagged liposomes did increase with time, indicating that Tf-tagged liposomes may also be endocytosed via a different process than receptor mediated endocytosis. Although we have not been able to determine which mechanisms were involved, we consistently found a higher HRP activity after incubation with lipo Tf than after incubation with lipo C.

Intracellular trafficking of Tf-tagged liposomes

The binding of Tf-tagged liposomes is 10–fold higher than the association (figure 1B). This may be explained by intracellular degradation of HRP. This phenomenon becomes apparent, since we determine the activity of HRP rather than the amount of

endocytosed HRP. From literature it is known that liposomes are intracellularly routed towards the lysosomes (23). In these lysosomes HRP (and other proteins) are subject to degradation (8, 9). Normally, the TfR-Tf complex is not directed towards lysosomes, but recycles from endosomes back to the cell surface (24, 25). However, when Tf is attached to a 100 nm liposome it has not been described what the effect is on the intracellular trafficking of the TfR-Tf-liposome complex.

To determine the mechanism of endocytosis of Tf-tagged liposomes, we have applied phenylarsine oxide (inhibitor of clathrin-coated vesicles associated with the TfR) and indomethacin (inhibitor of caveolae formation) (6). However, these inhibitors did not have a conclusive effect. We expected that by inhibition of endocytosis we would find a higher HRP activity, since HRP would not be intracellularly degraded. However, this was the case in one experiment, but not in another experiment with a different batch liposomes. Also, the use of chloroquine or NH_4Cl to prevent acidification of endosomes and therefore fusion with lysosomes did not have an effect on HRP activity after association of Tf-tagged liposomes by BCEC. Therefore, it was not possible to determine the mechanism of endocytosis.

Although we have incubated BCEC in the presence of endogenous Tf, we have tried to use an even higher excess of free Tf to further block TfR-mediated endocytosis. BCEC were pre-incubated for 30 min with 0.5 – 5 mg/ml Tf in DMEM+S (0.4 mg/ml Tf) before incubation with liposomes (3 μg/ml HRP). No significant inhibition of association of Tf-tagged liposomes was detected (data not shown). Since higher concentrations of Tf showed a decrease in cellular protein, we have not increased the concentration of free Tf beyond 5 mg/ml. Therefore, we have not been able to show that Tf-tagged liposomes are exclusively endocytosed via a TfR mediated mechanism.

Comparison of Tf-tagged liposomes and Tf-HRP conjugates

The binding (4 °C) of Tf-tagged liposomes was higher than the association (37 °C, figure 1B). In contrast, the association of the Tf-HRP conjugates, which we have described recently was 5.2 ng HRP per mg cell protein (6) and was approximately 4-fold higher than the binding. For this BCEC were incubated for 2 hours with Tf-HRP conjugates, based on 3 μg/ml HRP. The association at 37 °C of Tf-HRP was

approximately 7.5–fold higher than the association of Tf-tagged liposomes. The experiments with Tf-HRP were performed in PBS without serum, in order to eliminate potential interference with endogenous Tf. However, experiments with liposomes were performed in DMEM+S containing endogenous Tf. We found that the addition of serum was essential to minimise non-specific association of non-tagged liposomes. Still, the difference between PBS and DMEM+S is unlikely to cause the large difference between association of the Tf-HRP conjugates and the Tf-tagged liposomes. Furthermore, the binding of Tf-tagged liposomes is higher than the association, which indicates that the difference in association between Tf-HRP and Tf-tagged liposomes is most likely not caused by differences in TfR binding and endocytosis, but to a difference in intracellular trafficking. Furthermore, we assume that the rate of internalization is slower for the larger Tf-tagged liposomes than for Tf-HRP conjugates.

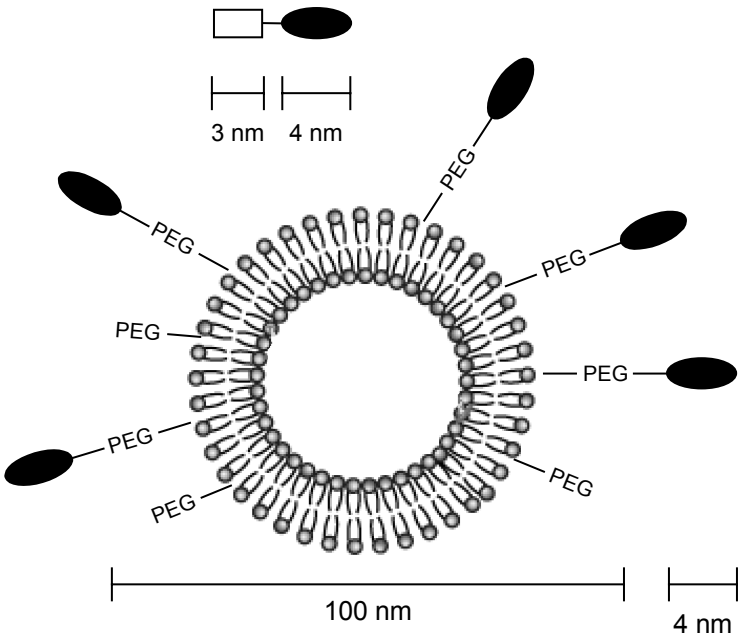


Figure 2: Schematic comparison between HRP encapsulated in a Tf-tagged liposome and a Tf-HRP conjugate. Tf (black oval) is approximately 4 nm (Enns and Sussman, 1981). HRP (square) has a similar size (approximately 3 nm (Ojteg *et al.*, 1987)), while a liposome is 100 nm in diameter. This picture is not to scale, the liposome should be 4 times larger for real comparison with Tf. Liposomes prepared for this research contained 63 – 74 Tf molecules per liposome.

The difference in association of Tf-tagged liposomes and Tf-HRP conjugates may be explained by the difference in size. HRP has a Stokes-Einstein radius of approximately 3 nm (26), and is thus much smaller than a 100 nm-liposome. In addition, the Stokes-Einstein radius of Tf is approximately 4 nm (27). Figure 2 gives a schematic representation for Tf-HRP conjugates and Tf-tagged liposomes. We hypothesise that the intracellular trafficking of Tf-HRP conjugates is similar to endogenous Tf, while the intracellular trafficking of the larger Tf-tagged liposomes is mainly determined by the liposomes. Tf and Tf-HRP are both endocytosed via clathrin-coated vesicles that are associated with receptor-mediated endocytosis (6, 13). For the endocytosis of HRP loaded Tf-tagged liposomes multiple processes, such as non-specific vesicular endocytosis (pinocytosis) may play a role. However, the high binding of Tf-tagged liposomes compared to non-tagged liposomes suggests that TfR mediated processes play a role.

Conclusions and Perspectives

In this paper we described the preparation, as well as the binding and association of Tf-tagged liposomes. Furthermore, we compared these results with the association of Tf-HRP conjugates. This enabled us to determine the differences in internalisation and intracellular trafficking of the protein drug. By using liposomes, we do not need to chemically modify the drug molecule as for the conjugates and after injection the drug is protected against degradation in serum. In addition, by using liposomes it is possible to circumvent the efflux-transporter P-glycoprotein (7) which is an advantage for drug targeting to the brain.

We found that the binding at 4 °C of Tf-tagged liposomes is higher than the association at 37 °C. We assume that this difference is caused by intracellular degradation of HRP. Therefore, it is important to realise that we have determined the HRP activity, rather than the amount of HRP. By doing so, we obtain a more realistic value for association of an active protein or enzymatic drug. Alternatively, Mumtaz and Bachhawat (28) have shown that stabilisation of HRP with dextran improves the intracellular stability of HRP. They have incorporated dextran-HRP into liposomes to target the lysosomes for the treatment of metabolic disorders, such as lysosomal storage

disease. Other information about differences in intracellular trafficking routes were published by Beyer *et al* (29). They have found that liposomal doxorubicin showed less endocytosis than free doxorubicin, but that the intracellular distribution was similar to free doxorubicin. In contrast, Tf- and BSA-doxorubicin conjugates were differently distributed. These findings are similar to our results and could imply that depending on the intracellular target, a Tf-drug conjugate or a Tf-tagged liposome should be used.

The association of Tf-tagged liposomes is significantly higher than non-tagged liposomes. Schreier *et al* (30) have shown that the lipid composition of the liposome can influence the intracellular trafficking. Furthermore, the size of liposomes can determine the route of uptake (31, 32). Although we were not able to determine the exact mechanism of endocytosis and intracellular fate of Tf- and non-tagged liposomes, it is still possible to increase the intracellular delivery of drugs by using Tf-tagged liposomes. Over the years, new techniques have been developed which allow release of the liposomal content in the cytosol, rather than in the lysosomes. For example, by using pH-sensitive liposomes the intra-endosomal and intra-lysosomal degradation of the encapsulated drug can be avoided. Soon after internalisation these liposomes fall apart when the endosomal pH decreases, thereby releasing their content. Subsequent endosomal escape might be induced by adding pH dependent fusogenic peptides, such as GALA (33, 34).

In conclusion, targeting liposomes encapsulating protein or peptide drugs to the TfR on BCEC is possibly limited due to intracellular degradation of the protein drug. However, as not all drugs are susceptible to degradation, it may be an advantage to target liposomal contents to the lysosomes for those drugs that can withstand the harsh conditions in the lysosomes (e.g. to treat lysosomal storage disease). Furthermore, our experiments suggest that liposomes release some of their content within the BBB, making targeting of liposomes to the TfR on BCEC an attractive approach for brain drug delivery.

Acknowledgements

The authors thank Jos van Brussel for the AAS measurement. This work was financially supported by grant 014-80-003 from STIGO (stimulation for innovative drug research, ZonMw), the Hague, the Netherlands and grant 10F02.17 from the Hersenstichting (Brain Foundation), the Hague, the Netherlands.

References

1. Rubin, L. L. and Staddon, J. M. (1999) The cell biology of the blood-brain barrier. *Annu Rev Neurosci* **22** 11-28
2. Pardridge, W. M. (1999) Blood-brain barrier biology and methodology. *J Neurovirol* **5** (6): 556-569
3. Pardridge, W. M. (2002) Drug and gene delivery to the brain: the vascular route. *Neuron* **36** (4): 555-558
4. Pardridge, W. M. (1998) CNS drug design based on principles of blood-brain barrier transport. *J Neurochem* **70** (5): 1781-1792
5. Shi, N., Boado, R. J. and Pardridge, W. M. (2001) Receptor-mediated gene targeting to tissues in vivo following intravenous administration of pegylated immunoliposomes. *Pharm Res* **18** (8): 1091-1095
6. Visser, C. C., Stevanović, S., Voorwinden, L. H., Gaillard, P. J., Crommelin, D. J. A., Danhof, M. and de Boer, A. G. (2004) Validation of the transferrin receptor for drug targeting to brain capillary endothelial cells *in vitro*. *J Drug Target* **12** (3): 145-150
7. Huwyler, J., Cerletti, A., Fricker, G., Eberle, A. N. and Drewe, J. (2002) By-passing of P-glycoprotein using immunoliposomes. *J Drug Target* **10** (1): 73-79
8. Banks, W. A. and Broadwell, R. D. (1994) Blood to brain and brain to blood passage of native horseradish peroxidase, wheat germ agglutinin, and albumin: pharmacokinetic and morphological assessments. *J Neurochem* **62** (6): 2404-2419
9. Broadwell, R. D. (1989) Transcytosis of macromolecules through the blood-brain barrier: a cell biological perspective and critical appraisal. *Acta Neuropathol (Berl)* **79** (2): 117-128
10. Iden, D. L. and Allen, T. M. (2001) In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. *Biochim Biophys Acta* **1513** (2): 207-216
11. Moreira, J. N., Ishida, T., Gaspar, R. and Allen, T. M. (2002) Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity. *Pharm Res* **19** (3): 265-269
12. Gaillard, P. J., Voorwinden, L. H., Nielsen, J. L., Ivanov, A., Atsumi, R., Engman, H., Ringbom, C., de Boer, A. G. and Breimer, D. D. (2001) Establishment and functional characterization of an in

vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur J Pharm Sci* **12** (3): 215-222

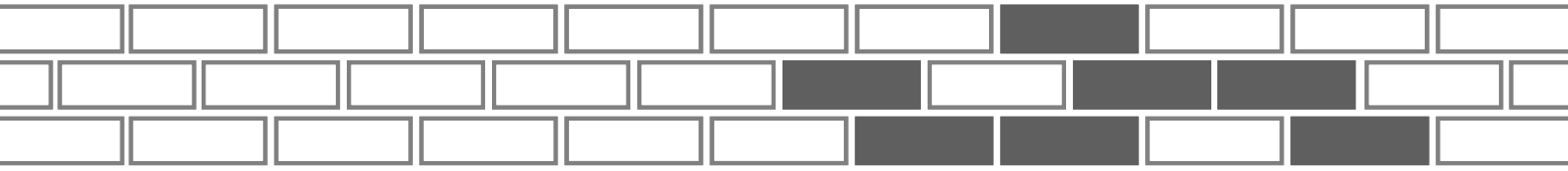
13. Visser, C. C., Voorwinden, L. H., Crommelin, D. J. A., Danhof, M. and de Boer, A. G. (2004) Characterization and modulation of the transferrin receptor on brain capillary endothelial cells. *Pharm Res* **21** (5): 761-769
14. Visser, C. C., Voorwinden, L. H., Harders, E. R., Eloualid, M., van Bloois, L., Crommelin, D. J. A., Danhof, M. and de Boer, A. G. Coupling of metal containing homing devices to liposomes via a maleimide linker: use of TCEP to stabilize thiol-groups without scavenging metals. *J Drug Target, in press*
15. Fukushima, H., Iwasaki, M., Yosie, O. and Igarashi, H. (2001) Induction of reduced endothelial permeability to horseradish peroxidase by factor(s) of human astrocytes and bladder carcinoma cells: detection in multi-well plate culture. *Methods Cell Sci* **23** (4): 211-219
16. Rouser, G., Fkeischer, S. and Yamamoto, A. (1970) Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5** (5): 494-496
17. Crommelin, D. J. (1984) Influence of lipid composition and ionic strength on the physical stability of liposomes. *J Pharm Sci* **73** (11): 1559-1563
18. Corvo, M. L., Jorge, J. C., van't Hof, R., Cruz, M. E., Crommelin, D. J. and Storm, G. (2002) Superoxide dismutase entrapped in long-circulating liposomes: formulation design and therapeutic activity in rat adjuvant arthritis. *Biochim Biophys Acta* **1564** (1): 227-236
19. Vingerhoeds, M. H., Steerenberg, P. A., Hendriks, J. J., Crommelin, D. J. and Storm, G. (1996) Targeted delivery of diphtheria toxin via immunoliposomes: efficient antitumor activity in the presence of inactivating anti-diphtheria toxin antibodies. *FEBS Lett* **395** (2-3): 245-250
20. Mastrobattista, E., Crommelin, D. J., Wilschut, J. and Storm, G. (2002) Targeted liposomes for delivery of protein-based drugs into the cytoplasm of tumor cells. *J Liposome Res* **12** (1-2): 57-65
21. Tirumalai, R. S., Chan, K. C., Prieto, D. A., Issaq, H. J., Conrads, T. P. and Veenstra, T. D. (2003) Characterization of the low molecular weight human serum proteome. *Mol Cell Proteomics* **2** (10): 1096-1103
22. Baker, E. and Morgan, E. (1994) Iron Transport. In *Iron Metabolism in Health and Disease* (J. Brock, J. Halliday, M. Pippard and L. Powell, eds.), Saunders, London, pp. 63-95
23. Gregoriadis, G. (1995) Engineering liposomes for drug delivery: progress and problems. *Trends Biotechnol* **13** (12): 527-537
24. Broadwell, R. D., Baker-Cairns, B. J., Friden, P. M., Oliver, C. and Villegas, J. C. (1996) Transcytosis of protein through the mammalian cerebral epithelium and endothelium. III. Receptor-mediated transcytosis through the blood-brain barrier of blood-borne transferrin and antibody against the transferrin receptor. *Exp Neurol* **142** (1): 47-65
25. Moos, T. and Morgan, E. H. (2000) Transferrin and transferrin receptor function in brain barrier systems. *Cell Mol Neurobiol* **20** (1): 77-95
26. Ojteg, G., Nygren, K. and Wolgast, M. (1987) Permeability of renal capillaries. I. Preparation of neutral and charged protein probes. *Acta Physiol Scand* **129** (3): 277-286
27. Enns, C. A. and Sussman, H. H. (1981) Physical characterization of the transferrin receptor in human placentae. *J Biol Chem* **256** (19): 9820-9823

28. Mumtaz, S. and Bachhawat, B. K. (1992) Enhanced intracellular stability of dextran-horse radish peroxidase conjugate: an approach to enzyme replacement therapy. *Biochim Biophys Acta* **1117** (2): 174-178
29. Beyer, U., Rothern-Rutishauser, B., Unger, C., Wunderli-Allenspach, H. and Kratz, F. (2001) Differences in the intracellular distribution of acid-sensitive doxorubicin-protein conjugates in comparison to free and liposomal formulated doxorubicin as shown by confocal microscopy. *Pharm Res* **18** (1): 29-38
30. Schreier, H., Moran, P. and Caras, I. W. (1994) Targeting of liposomes to cells expressing CD4 using glycosylphosphatidylinositol-anchored gp120. Influence of liposome composition on intracellular trafficking. *J Biol Chem* **269** (12): 9090-9098
31. Conner, S. D. and Schmid, S. L. (2003) Regulated portals of entry into the cell. *Nature* **422** (6927): 37-44
32. Rejman, J., Oberle, V., Zuhorn, I. S. and Hoekstra, D. (2004) Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J* **377** (Pt 1): 159-169
33. Kakudo, T., Chaki, S., Futaki, S., Nakase, I., Akaji, K., Kawakami, T., Maruyama, K., Kamiya, H. and Harashima, H. (2004) Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system. *Biochemistry* **43** (19): 5618-5628
34. Parente, R. A., Nir, S. and Szoka, F. C., Jr. (1990) Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry* **29** (37): 8720-8728

Section 4

Liposomal drug delivery under inflammatory disease conditions

Chapter 7



Interaction of liposomes with lipopolysaccharide: influence of time, serum and liposome composition

Submitted for publication

C.C. Visser, L.H. Voorwinden, L. van Bloois[*], P.J. Gaillard, M. Danhof, D.J.A. Crommelin[*], and A.G. de Boer

Leiden/Amsterdam Centre for Drug Research (LACDR), Leiden University, Division of Pharmacology, PO Box 9502, 2300 RA Leiden, The Netherlands

[*]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), PO Box 80082, 3508 TB Utrecht, The Netherlands

Abstract

The central nervous system is protected by the blood-brain barrier (BBB). Under inflammatory conditions (such as bacterial meningitis) brain homeostasis is changed as a result of a disrupted BBB. This may be caused in part by lipopolysaccharide (LPS), that has many effects, among which the formation of free radicals. These free radicals cause a disruption of the tight junctions between brain capillary endothelial cells (BCEC). We have investigated whether liposomes are able to scavenge LPS by measuring the TransEndothelial electrical resistance as a measure of BBB tightness. After 6 hours of pre-incubation of LPS and liposomes (egg phosphatidylcholine (EPC-35) and cholesterol, with 0, 5 or 10 % polyethylene glycol (PEG-PE)), the opening of the BBB *in vitro* was delayed and less pronounced compared to LPS that was not pre-incubated. However, since the functional read-out of the effect of LPS in the *in vitro* BBB model was highly variable, it was not possible to quantify this liposomal scavenging effect by a functional assay. Therefore, we have chosen to investigate this physico-chemically. We found that liposomes, consisting of EPC-35 alone (least stable) or of EPC-35 and cholesterol with 5 or 10 % PEG-PE (more stable) are able to scavenge fluorescently labelled LPS in a time dependent manner. The surface density of PEG or the absence or presence of serum (10 %) did not influence the ability of liposomes to scavenge LPS.

We conclude that liposomes used for the targeting of drugs under inflammatory disease conditions are able to scavenge LPS in a time dependent manner.

Introduction

The central nervous system is protected by the blood-brain barrier (BBB). This barrier maintains homeostasis in the brain, by minimising paracellular and transcellular transport across the endothelial cells (1). The main barrier is formed by brain capillary endothelial cells (BCEC), which are stimulated by astrocytic endfeet (2). Between BCEC, so called tight junctions, are formed. Lipopolysaccharide (LPS) can induce an opening of the tight junctions and thereby decrease BBB functionality. LPS induces transcription of acute phase proteins and activates protein kinases (3). Furthermore, Gaillard *et al* (4) have shown that LPS induces the formation of free radicals, which in turn cause an opening of the tight junctions. By pre-incubation of BCEC with high concentrations of the radical scavenger N-acetyl-L-cysteine (NAC), opening of the BBB by LPS *in vitro* was prevented (4). However, NAC is very hydrophilic and, therefore, poorly penetrates the cellular membrane. Incorporation of NAC into the aqueous compartment of liposomes can potentially increase the intracellular delivery of NAC. We have therefore prepared Tf-tagged liposomes, consisting of EPC-35 and cholesterol, encapsulating NAC. After overnight pre-incubation of the *in vitro* BBB these liposomes were able to counteract LPS induced BBB opening (unpublished results). However, liposomes that did not contain NAC also showed protection of the BBB against LPS after overnight pre-incubation. From literature it is known that LPS, when incorporated into liposomes, is still endocytosed by macrophages, but has a reduced potency to induce tumour cytotoxicity and tumor necrosis factor (TNF) secretion (5). In addition, it has been shown that lipoproteins in serum have the ability to scavenge LPS (6).

We have investigated whether liposomes can scavenge LPS by pre-incubation of liposomes and LPS before addition to the *in vitro* BBB model. After at least 6 hours of pre-incubation with liposomes, the effect of LPS on the *in vitro* BBB model was reduced. However, as was observed earlier by Gaillard *et al* (4), the LPS effect on the *in vitro* BBB model was found to be variable. In addition, the effect of the combination of liposomes and LPS on the *in vitro* BBB model was variable, which made it difficult to quantify these effects by a functional read-out. Therefore, we have chosen to extend our investigations by a physico-chemical approach.

For these experiments we have incubated fluorescently labelled LPS (LPS-Alexa) with liposomes for 2, 6 or 16 hours and separated the liposomes and LPS micelles on a

Sepharose CL4B column. We have performed incubations in the absence or presence of serum (10 %) and with different polyethylene glycol-2000 (PEG) densities on the surface of the liposomes. This method enabled us to directly measure the capacity of liposomes to scavenge LPS and to relate this to the results we have found in our *in vitro* BBB model.

Experimental

Liposomes

Liposomes of egg phosphatidylcholine (EPC-35) only or EPC-35 and cholesterol (2:1 molar ratio) were prepared by hydration of a lipid film in HEPES buffered saline (HBS), pH 7.0, at a phospholipid concentration of 18 μM. Liposomes of EPC-35 and cholesterol were stabilised with 0, 5 or 10 % PEG-PE After hydration of the lipid film, liposomes were extruded 8 times through 200 and 100 nm polycarbonate filters with a hand extruder (Alabaster, AL, USA) to obtain a homogeneous dispersion of liposomes. The final phospholipid content was determined according to Rouser (7). Liposomal size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd. Malvern, UK). The polydispersity index (p.i.) was used as an indication for the size distribution. The p.i. can range from 0 (monodisperse) to 1 (polydisperse).

LPS effect in the in vitro BBB model

The preparation of the *in vitro* BBB model was described previously (8). To characterise the effect of LPS on the *in vitro* BBB model the TransEndothelial electrical resistance (TEER) was measured, as described by Gaillard et al (4).

LPS was pre-incubated at 37 °C for 2, 6 or 16 hours with liposomes (1: 750 weight ratio) in PBS (0.11 mM KH_2PO_4, 0.56 mM Na_2HPO_4 and 150 mM NaCl) without serum. Subsequently, the LPS-liposome mixture was added to the apical side of the *in vitro* BBB model, obtaining a final concentration of 100 ng/ml LPS. The cells in the *in vitro* BBB model were cultured in the presence of 10 % serum and this medium was not changed before addition of LPS-liposome mixtures. TEER was measured each hour and was corrected for background and surface area of a transwell filter. TEER is expressed as a percentage of control (PBS).

Association of fluorescent LPS to liposomes

Fluorescently labelled LPS (LPS-Alexa, 400 ng) was incubated with 400 nmol of liposomes (1:750 weight ratio) in 200 µl PBS for 2, 6 or 16 hour. Incubation was performed in the absence or presence of 10 % serum, at 37 °C. After incubation, 150 µl of the liposome-LPS mixture was separated on a Sepharose CL4B column. Elution was based on gravity, and fractions of approximately 0.5 ml were collected every 2 minutes. Between each separation the column was rinsed with PBS for at least 30 minutes; overnight the column was rinsed with 20 % ethanol.

Fractions were analysed for LPS-Alexa by fluorescence analysis (Fluostar Optima, BMG Labtech, Offenburg, Germany). The LPS-liposome incubation mixture that was not separated was diluted to a standard curve of 0 – 0.4 ng/ml LPS-Alexa. Excitation was set at 480 nm, emission at 530 nm and each well was scanned with 20 flashes in the matrix mode. The emission wavelength of LPS-Alexa was 519 nm according to Molecular probes. However, we obtained more reproducible results with a 530 nm emission wavelength filter. The 530 nm filter had a smaller range (12 nm), while the filter for the 520 nm wavelength had a broad range (30 nm). We obtained a full absorption- and emission spectrum for LPS-Alexa, which showed an excitation maximum at 495 nm and an emission maximum at 520 nm. The gain was 3173 ± 43 for each plate that was measured. The gain is set to the well containing the highest concentration fluorescent label (in our case LPS-Alexa) and is used to automatically recalculate the measured value to the fluorescent intensity. Each curve was corrected for its own PBS background, which was 1967 ± 838.

The AUC of each fluorescence peak was determined with the trapezium rule, in which the average LPS concentration in two following samples was multiplied by the time interval between those samples. Initially in this research black fluorescent 96-wells plates from 2 suppliers were compared. No differences were found in the results.

To determine which fractions contained the liposomes, an enzymatic phospholipid assay was performed on fraction numbers 5 – 15, 17, 20, 22 and 24.

After each incubation and separation, the LPS-Alexa micelles were present in the same fractions, independent of time of incubation and liposomes present during the incubation. Furthermore, for LPS alone, for LPS + EPC-35 (2 h, in the presence of serum) and for LPS + liposomes with 5 % PEG-PE (16 h, in the absence of serum) we have repeated the incubation and separation to verify our results. The variation between the two occasions of each sample was less than 5 %.

Materials

LPS-Alexa (serotype 055:B5) was obtained from Molecular Probes (Leiden, the Netherlands). EPC-35 was purchased from Lipoid GmbH (Ludwigshafen, Germany) and PEG_{2000}-DSPE from Avanti Polar Lipids Inc. (Alabaster, AL, USA). LPS (serotype 055:B5) and cholesterol were obtained from Sigma (Zwijndrecht, the Netherlands) and Sepharose CL4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Fetal calf serum from BioWhittaker Europe (Verviers, Belgium), while other cell culture media were obtained from BioWhittaker Europe (Verviers, Belgium). Transwell polycarbonate filters (surface area 0.33 cm^2, pore size 0.4 µm) and black fluorescent 96-wells plates

were obtained from Corning Costar (Cambridge, MA, USA). Black fluoresecent 96-wells plates were also obtained from Greiner Bio-one GmbH (Frickenhausen, Germany). Phospholipid B reagent was obtained from Wako chemicals GmbH (Neuss, Germany).

Results

The liposomes that were prepared contained 12.5 – 15.4 mM phospholipid, while liposomes with 5 and 10 % PEG-PE also contained cholesterol. Table I summarises the liposome characteristics. The liposomal size ranged from 124 to 135 nm in diameter and the polydispersity index ranged from 0.07 to 0.12. The size increased to approximately 140 nm in 2 weeks, but did not change thereafter. The polydispersity index increased slightly to 0.14, indicating that liposomes, even after 2 weeks, had a narrow particle size distribution.

LPS was pre-incubated for 2, 6 or 16 hours with liposomes. The functional read-out (i.e. measuring the TEER of the *in vitro* BBB model) was used to estimate the effect of the interaction between liposomes and LPS. Figure 1 shows some typical graphs of the effect of LPS on TEER. The effect of LPS on TEER is variable: in figure 1B, for example, TEER is decreased after addition of LPS, but shows a recovery at 3 hours, while in figure 1A, LPS decreases TEER without a subsequent recovery of TEER. The effect of 6 hours pre-incubation with liposomes was also variable between experiments. Figure 1A shows a delayed and less steep decrease in TEER, while figure 1B shows a less intense TEER decrease at 2 hours, but no difference between LPS and LPS with liposomes at later time-points. Figure 1C shows that LPS which is 6 hours pre-incubated without liposomes at 37 °C does not show a difference compared to LPS that is added directly to the *in vitro BBB* model.

Table I: Characteristics of liposomes, with regards to their phospholipid concentration, size and polydispersity index (p.i., a p.i. < 0.2 indicates as rather narrow particle size distribution). The final column indicates for which experiment the liposomes are used.

liposomes	PEG-PE	phospholipid (mM)	size (nm)	p.i.	experiment
EPC-35 : cholesterol	5 %	18.1	154	0.07	BBB model
EPC-35 : cholesterol	5 %	10.5	156	0.07	fig. 1A
EPC-35 : cholesterol	-	6.9	160	0.05	BBB model
EPC-35 : cholesterol	5 %	10.3	158	0.06	fig. 1B
EPC-35 : cholesterol	10 %	7.5	163	0.08	
EPC-35	-	15.4	124	0.12	LPS-Alexa
EPC-35 : cholesterol	5 %	12.5	135	0.07	fig. 2
EPC-35 : cholesterol	10 %	14.2	125	0.08	

Subsequently, a physico-chemical approach was applied to quantify the interaction of liposomes and LPS. Liposomes were incubated with LPS-Alexa at 37 °C for 2, 6 or 16 hours. Figure 2 shows the elution profile of LPS-Alexa and EPC-35 liposomes after separation of the liposome-LPS mixture on a Sepharose CL4B column. When LPS-Alexa alone was applied on the Sepharose CL4B column a fluorescence peak between 22 and 50 minutes was visible, while after pre-incubation with liposomes also a fluorescence peak between 16 and 22 minutes was visible. For the calculation of the LPS concentration a standard curve of 0 - 0.4 ng/ml LPS-Alexa was used. The presence of liposomes did not affect fluorescence measurement as is shown from the standard curves in the absence or presence of EPC-35 (figure 3). The elution profiles of the other LPS-liposome mixtures were similar (data not shown).

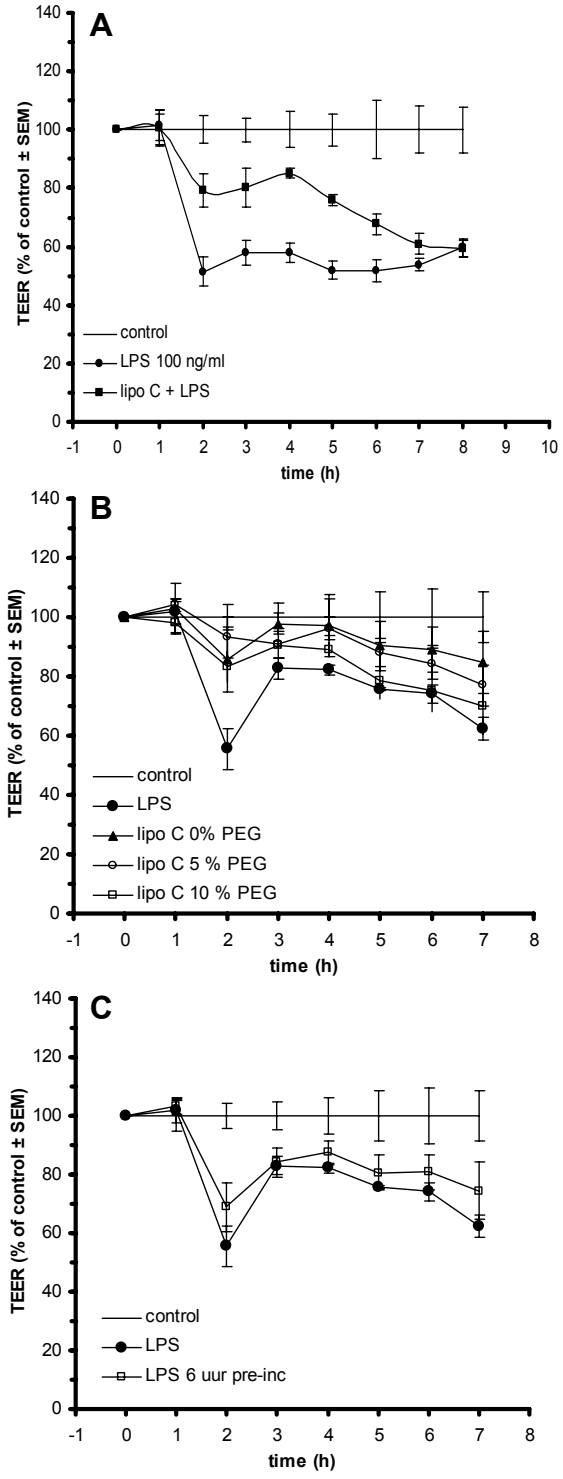


Figure 1: LPS and liposomes were pre-incubated for 6 hours after which TEER was measured up to 8 hours after addition to the *in vitro* BBB model. Figure A shows a delayed and less intense decrease in TEER after pre-incubation of LPS with liposomes containing 5% PEG-PE (lipo C) and transferrin (lipo T). Figure B only shows a less intense decrease at 2 hours after pre-incubations with liposomes containing 0, 5 or 10 % PEG-PE. Figure C shows that 6 hours of pre-incubation at 37 °C without liposomes does not affect the LPS effect itself. The graph in figure B and C are from the same experiment and have therefore the same LPS and control curve.

Phospholipid determination showed that the liposomes eluted in peak 1 (between 16 and 22 min), while no phospholipid was detected in peak 2 (between 22 and 50 min). This indicates that the fluorescence in peak 1 is liposome associated LPS. The AUC of both peaks was determined and the LPS that was associated with the liposomes was calculated as the percentage AUC_{peak1} of the total AUC of both peaks (table II). The association of LPS with liposomes was shown to be time dependent (table II). The absence or presence of 10 % serum did not change the association of liposomes and LPS. When LPS was incubated overnight at 37 °C in the presence of serum only a small peak was detected between 16 and 22 min (figure 2C). The AUC of this peak was only 2 % of the total AUC.

Discussion

Bacterial meningitis and sepsis can affect the BBB (9). This is mainly caused by LPS from gram negative bacteria. One of the effects of LPS is the formation of free radicals, which in turn cause an opening of the tight junctions between BCEC (4). From literature it is known that the effect of LPS on tumour cytotoxicity and TNF secretion diminishes when it is incorporated into liposomes (5). In these investigations both free LPS and liposomal LPS were endocytosed by macrophages, but liposomal LPS showed a diminished effect. We have investigated whether liposomes can scavenge LPS, thereby decreasing the effect on the BBB *in vitro*. For this we have used liposomes of EPC-35 and cholesterol with 0, 5 or 10 % PEG-PE. Initially, LPS was incubated with liposomes (1:750 weight ratio) in PBS without serum at 37 °C for 2, 6 or 16 hours. After incubation, the mixture was added to the *in vitro* BBB model (containing 10 % serum), developed by Gaillard (8) and the TEER was measured every hour up to 8 hours as a measure of BBB functionality (figure 1). The *in vitro* BBB model is a well characterised and established model for BBB transport (8). However, the effect of LPS on this model shows variability in the initial effect on TEER, as well as on the recovery phase. This was also observed by Gaillard *et al* (4). The high variability in LPS effect made it difficult to quantify the effect of the liposomes on LPS. Figure 1A shows a clearly delayed and less intense effect of LPS after pre-incubation with liposomes, while figure 1B only shows an effect of liposomes on LPS at 2 hour after addition of the LPS-liposome mixture to the *in vitro* BBB model. Pre-incubation of LPS at 37 °C without liposomes did

not affect the LPS effect in the *in vitro* BBB model (figure 1C). Therefore, the changed effect of LPS on the *in vitro* BBB model was due to the pre-incubation with liposomes. However, due to the high variability of the results obtained with this model, we have chosen for a purely physico-chemical approach to assess the interaction between LPS and liposomes that was hypothesised to be the basis of the "neutralising" effect by liposomes. For this fluorescently labelled LPS was used. Separation of liposomes from LPS was performed on a Sepharose CL4B column. This method was used previously to separate liposomes from PEG-PE micelles (10). LPS can form aggregates or micelles as well. For LPS from E.coli 055:B5 a crititical aggregation concentration of 38 μg/ml is reported (11). We have used a final concentration of 2 μg/ml LPS from the same serotype, at which no micelles or aggregates are formed. At this concentration a clear separation between liposomes and LPS on the Sepharose CL4B column was observed (figure 2).

Table II: The AUC of the liposomal peak and the AUC of the LPS peak were calculated using the trapezium rule. The fluorescently labelled LPS associated with liposomes is represented as a percentage of the total AUC. Each incubation and separation was performed only once.

	without serum			with serum (10%)		
	2 hours	6 hours	16 hours	2 hours	6 hours	16 hours
EPC-35	10	19	23	15	21	21
5 % PEG-PE	7	n.d.*	20	12	15	12
10 % PEG-PE	10	16	21	10	22	32

* not determined

For this research we have used liposomes of EPC-35, which are not very stable in the presence of serum. In addition, the liposomal bilayer was stabilised by cholesterol (EPC-35: cholesterol is 2:1) and stealth properties were induced in the liposomes by the addition of PEG-PE. In general, liposomes with 5 % PEG-PE are used for *in vivo* drug delivery to prevent liposomes from elimination from the bloodstream. In our previous work we also used liposomes with 5 % PEG-PE for drug targeting *in vitro* (10). Liposomal size was approximately 130 nm in diameter and the polydispersion index was approximately 0.1 which indicates a narrow size distribution.

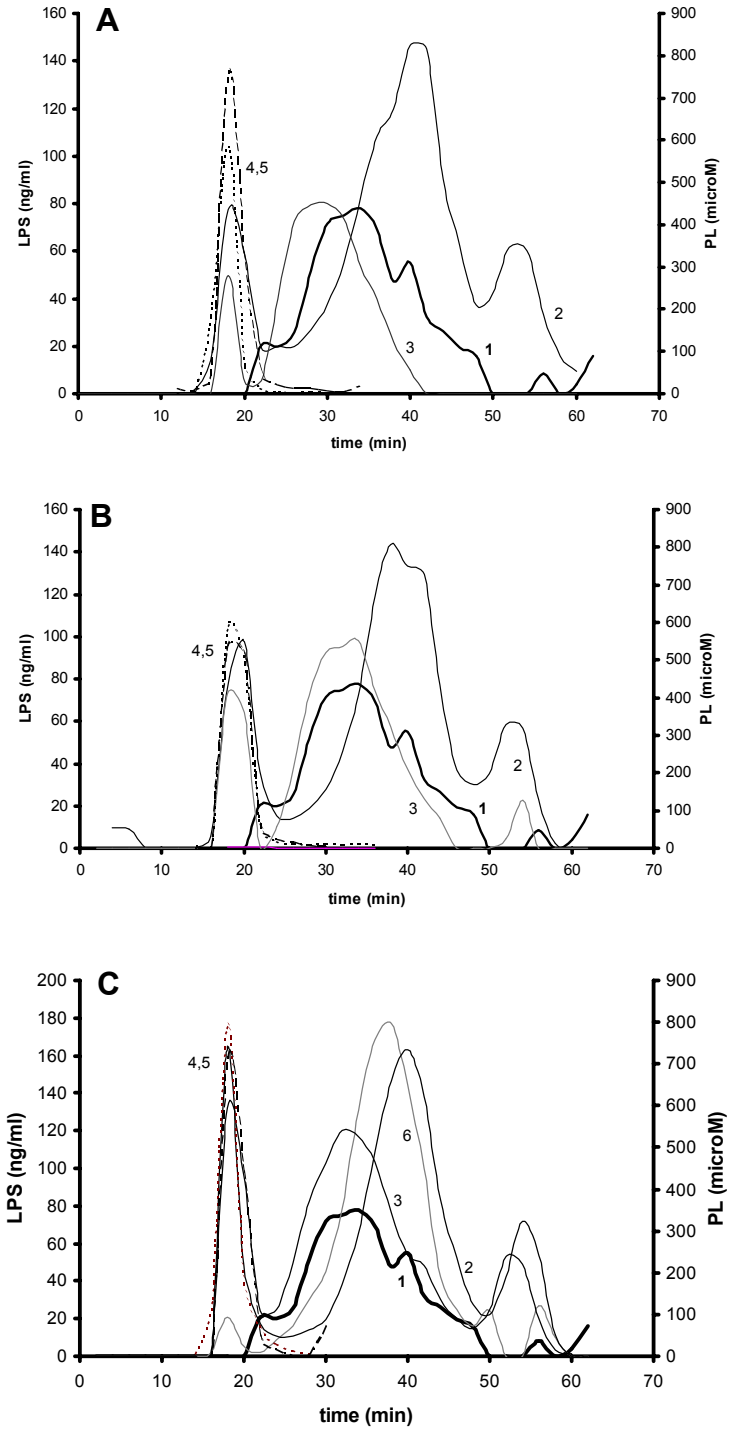


Figure 2: Liposomes of EPC-35 were pre-incubated with fluorescently labelled LPS in the absence or presence of 10 % serum for 2 hours (A), 6 hours (B), or 16 hours (C) before separation on a Sepharose CL4B column. Solid lines is the LPS concentration in ng/ml (left y-axis), dotted lines is the phospholipid (PL) concentration in μM (right y-axis). In each graph lines represent an elution profile of (1) LPS, which is not pre-incubated, (2) LPS + EPC-35 with 10 % serum, (3) LPS + EPC-35 without serum, (4) PL curve of LPS + EPC-35 with 10 % serum, (5) PL curve of LPS + EPC-35 without serum. Figure C also represents an elution profile of LPS pre-incubated with serum (6).

LPS-Alexa was incubated with liposomes (1:750 weight ratio) at 37 °C for 2, 6 or 16 hours. The liposomal peak was separated from the LPS peak by one or two fractions (figure 2), although in some samples overlap of the peaks was seen. The size distribution of the LPS was probably broad, since a broad fluorescent peak was visible. It was not possible to determine size and p.i. of the fluorescent LPS, since we could not prepare a high enough concentration of LPS-Alexa. For LPS of the same serotype, but without a fluorescent label we obtained a diameter of 40 nm and a p.i. of 0.4 at a LPS concentration of 1 mg/ml, as measured by dynamic light scattering. The relatively high p.i. indicates a broad particle size distribution. Sonication of the LPS solution had no effect on the size distribution as determined by fluorescence analysis after separation on a Sepharose-CL4B column (data not shown). The presence of the liposomes did not change the fluorescent signal as is shown from the standard curves diluted from the incubation mixtures with or without liposomes (figure 3).

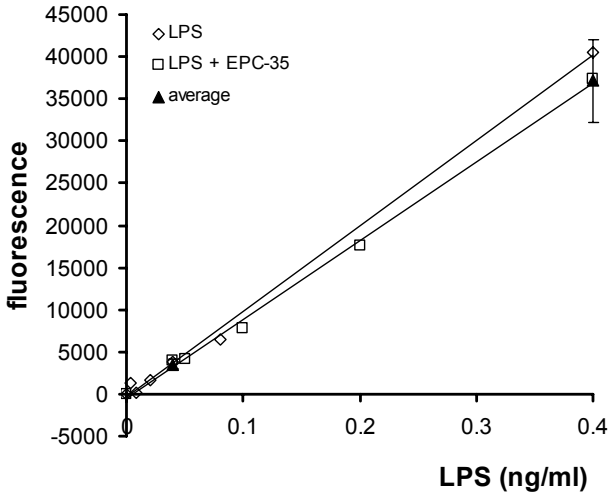


Figure 3: Standard curve of LPS-Alexa and LPS-Alexa in the presence of liposomes. At 0.04 and 0.4 ng/ml LPS-Alexa the averages (± s.d.) of all samples are represented. The slopes of the standard curves are $86*10^3 \pm 8*10^3$ (mean ± s.d.), the interception with the y-axis was 0.

With an enzymatic phospholipid assay it was determined which peak contained the liposomal fraction. LPS also contains a lipid tail which could be measured by a phospholipid determination, but the concentration was too low to be detected under the chosen conditions. Therefore, the phospholipid determination was representative for the liposomal fractions. Calculating the AUC with the trapezium rule revealed that for

liposomes consisting of EPC-35 the association of LPS was time dependent, but not dependent on the presence of 10 % serum (table II). We assumed that serum might have an inhibitory effect, but, in contrast, it had a slightly increasing effect on the association of LPS by liposomes. Wurfel and Wright (12) have reported that LPS-binding protein enhances the interaction of LPS with the phospholipid content of lipoproteins. This may account for the higher association of LPS by liposomes in the presence of serum, since LPS-binding protein is present in serum. Incubation in the presence of serum caused a shift to the right for the peak containing the LPS micelles (figure 2). This may be explained by the fact that lipoproteins present in serum have a diameter ranging from 5 - 12 nm for HDL to 30 – 80 nm for VLDL and are able to scavenge LPS (13). Therefore, a re-distribution in LPS particles or aggregates might have caused the shift in the peak containing the LPS micelles. It was shown that LPS that was pre-incubated in the presence of 10 % serum for 16 hours gave a small peak between 16 and 22 min. However, the AUC of this peak was only 2 % of the total AUC, indicating that serum does not interfere with the liposome-LPS interaction.

The composition of the liposomes, as well as the surface density of PEG-PE did not interfere with the ability of liposomes to interact with LPS (table II). In the *in vitro* BBB model we have found that after 6 hours pre-incubation of liposomes with LPS, the decrease in TEER was delayed and less pronounced, compared to LPS which was not pre-incubated. Gaillard *et al* (4) have shown that the effect on TEER was dependent on the LPS concentration. With the fluorescently labelled LPS we found that after 6 hours of incubation with liposomes, 15 - 20 % of the LPS was associated by liposomes (table II). Apparently, a decrease of 15 – 20 % in the "free" concentration LPS is already enough to alter the effect on the BBB *in vitro* (figure 1). We have not determined whether LPS was inserted into the bilayer or attached on the outer surface of the liposomes. However, since the liposomes with 5 and 10 % PEG-PE are sterically stabilised, it is unlikely that LPS is able to enter the liposomal bilayer. Although the position of the LPS on or in the liposomal bilayer is different, the decrease in LPS effect after pre-incubation with liposomes was comparable with results from Dijkstra *et al* (5). They have found that active incorporation of LPS into the liposomal bilayer did not affect the endocytosis of LPS, but reduced the effect of LPS on inflammatory mediators, such as tumour necrosis factor and interleukin-1.

For our work towards the delivery of liposomes encapsulating the free-radical scavenger NAC, the effect of liposomes on LPS is important. To discriminate between the effect of the drug and the effect of the liposomes on LPS-induced opening of the BBB *in vitro*, we have applied liposomes apically (directly in the BCEC compartment). In addition, LPS was added to the basolateral side of the transwell filter. By doing so, LPS and liposomes are in different compartments, thereby revealing the drug effect. However, the effects of LPS and NAC on the tightness of the BBB *in vitro* was too variable to draw solid conclusions about the effect of NAC.

In conclusion, in this short communication we have shown that liposomes are able to scavenge LPS in a time-dependent manner. The density of PEG-PE on the surface of the liposomes, as well as the absence or presence of serum had no effect on the ability of liposomes to scavenge LPS. These results are interesting for research that is performed with liposomes under inflammatory disease conditions, since the direct effect of liposomes on LPS may interfere with the interpretation of the effect of the drug that is incorporated into the liposomes.

Acknowledgements

This work was financially supported by grant 014-80-003 from STIGO (stimulation for innovative drug research, ZonMw), the Hague, the Netherlands and grant 10F02.17 from the Hersenstichting (Brain Foundation), the Hague, the Netherlands.

References

1. Pardridge, W. M. (1999) Blood-brain barrier biology and methodology. *J Neurovirol* **5** (6): 556-569
2. Rubin, L. L. and Staddon, J. M. (1999) The cell biology of the blood-brain barrier. *Annu Rev Neurosci* **22** 11-28
3. Baumann, H. and Gauldie, J. (1994) The acute phase response. *Immunol Today* **15** (2): 74-80
4. Gaillard, P. J., de Boer, A. B. and Breimer, D. D. (2003) Pharmacological investigations on lipopolysaccharide-induced permeability changes in the blood-brain barrier in vitro. *Microvasc Res* **65** (1): 24-31

5. Dijkstra, J., Larrick, J. W., Ryan, J. L. and Szoka, F. C. (1988) Incorporation of LPS in liposomes diminishes its ability to induce tumoricidal activity and tumor necrosis factor secretion in murine macrophages. *J Leukoc Biol* **43** (5): 436-444
6. Van Oosten, M., Rensen, P. C., Van Amersfoort, E. S., Van Eck, M., Van Dam, A. M., Breve, J. J., Vogel, T., Panet, A., Van Berkel, T. J. and Kuiper, J. (2001) Apolipoprotein E protects against bacterial lipopolysaccharide-induced lethality. A new therapeutic approach to treat gram-negative sepsis. *J Biol Chem* **276** (12): 8820-8824
7. Rouser, G., Fkeischer, S. and Yamamoto, A. (1970) Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5** (5): 494-496
8. Gaillard, P. J., Voorwinden, L. H., Nielsen, J. L., Ivanov, A., Atsumi, R., Engman, H., Ringbom, C., de Boer, A. G. and Breimer, D. D. (2001) Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur J Pharm Sci* **12** (3): 215-222
9. de Vries, H. E., Eppens, E. F., Prins, M., Kuiper, J., van Berkel, T. J., de Boer, A. G. and Breimer, D. D. (1995) Transport of a hydrophilic compound into the cerebrospinal fluid during experimental allergic encephalomyelitis and after lipopolysaccharide administration. *Pharm Res* **12** (12): 1932-1936
10. Visser, C. C., Stevanović, S., Voorwinden, L. H., van Bloois, L., Gaillard, P. J., Danhof, M., Crommelin, D. J. A. and de Boer, A. G. Targeting liposomes with protein drugs to the blood-brain barrier *in vitro. submitted for publication*
11. Aurell, C. A. and Wistrom, A. O. (1998) Critical aggregation concentrations of gram-negative bacterial lipopolysaccharides (LPS). *Biochem Biophys Res Commun* **253** (1): 119-123
12. Wurfel, M. M. and Wright, S. D. (1997) Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers: preferential interaction with particular classes of lipid. *J Immunol* **158** (8): 3925-3934
13. Van Amersfoort, E. S., Van Berkel, T. J. and Kuiper, J. (2003) Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin Microbiol Rev* **16** (3): 379-414

Section 5

General Discussion

Chapter 8

Summary, discussion and perspectives

Parts of this chapter will be published in an invited review

in Expert Opinion on Drug Delivery

Contents Chapter 8

1. Introduction

Drug delivery to the brain is limited due to the presence of the blood-brain barrier (BBB) (1). This barrier is located at the interface between blood and brain and its primary function is to maintain homeostasis in the central nervous system (CNS). Anatomically, the BBB is located at the brain capillary endothelial cells (BCEC), and its unique properties are induced by interactions with astrocytes, pericytes and neuronal cells (2, 3). This makes the BBB a highly dynamic organ.

The research described in this thesis focuses on the transferrin receptor (TfR) at the BBB for drug targeting and delivery to the brain. The TfR is abundantly expressed on BCEC, and is actively internalising its endogenous ligand, transferrin (Tf). For drug delivery purposes Tf-drug conjugates and Tf-tagged liposomes have been studied.

2. The transferrin receptor: target validation

To characterise and validate the TfR for drug targeting to the brain, primary cultured BCEC were used. These BCEC are the main component of the BBB and can be used for in vitro BBB research. To induce BBB-like properties, such as tight junctions and P-glycoprotein expression, BCEC can be co-cultured with astrocytes (4). However, for this research we have cultured the BCEC in astrocyte-conditioned medium (4) to be able to characterise the TfR more mechanistically.

The endogenous ligand, transferrin (Tf), was used for the characterisation of the TfR. To this end Tf was labelled with ^{125}I, to be able to quantify the receptor expression and internalisation (**chapter 3**). Table I summarises the TfR expression levels under physiological conditions (control), as well as in low (DFO) and high ($FeCl_3$) iron situations.

Table I: Total and extracellular expression, determined at 4 °C, of the TfR on primary cultured BCEC and the association of Tf, determined at 37 °C in the presence of DFO or $FeCl_3$ (1 mM). Data were analysed using NONMEM, K_d was estimated 2.4 ± 0.3 μg/ml and 5.0 ± 0.5 μg/ml for expression and association, respectively. Values for B_{max} are summarised in the table as mean (95 % confidence interval, CI). Intra-individual residual variation was determined with a proportional error model and was 10 % for expression levels and 9 % for association.

	B_{max} (fmol/μg cell protein)		
	total TfR expression	extracellular TfR expression	association
control	1.37	0.13	0.90
(CI)	(1.15 – 1.59)	(0.10 – 0.16)	(0.78 – 1.01)
DFO	3.68	0.63	2.31
(CI)	(2.73 – 4.63)	(0.55 – 0.71)	(2.03 – 2.59)
$FeCl_3$	1.64	not detectable	0.56
(CI)	(1.09 – 2.19)		(0.49 – 0.63)

The data in table I were analysed by nonlinear mixed effects modelling, allowing the estimation of a single unique value for the affinity (K_d) in conjunction with separate values for the receptor expression (B_{max}). Approximately 90 % of the receptors is stored in an intracellular pool, as can be seen from the difference between the high total TfR expression and the low extracellular TfR expression. After pre-incubation with the iron scavenger deferoxamine mesylate (DFO), both the expression as well as the association are increased. An excess of $FeCl_3$ has no effect on the total TfR expression, but lowers the association of Tf by the TfR. Therefore, we concluded that the TfR expression level was mainly regulated by the iron concentration in the cell culture medium. This was confirmed by literature reports (5-8). Furthermore, in the presence of the iron scavenger DFO, the increase in extracellular TfR expression was higher than the increase in total TfR expression. This indicates that not only new receptors are synthesised, but that there is also a shift from the intracellular pool to the extracellular membrane. The up-regulation of the TfR expression level constitutes in theory a basis for an increased targeting efficiency to this receptor. DFO (desferal®) is currently used for treatment of (transfusional) iron overload, but it has many side effects (9). *In vivo* upregulation of the TfR may still be possible when new iron chelators are developed. Furthermore, for the treatment of brain cancer, DFO could be used for its antiproliferative effects (10).

To determine the internalisation of the TfR, acid wash and trypsin cleavage techniques were used: with acid wash the extracellular bound Tf was removed, and with trypsin the extracellular TfR was cleaved at its trypsin-sensitive site. Results showed that approximately 70 – 80 % of the associated Tf is acid and trypsin resistant, indicating that most of the receptor-bound Tf is actively internalised within 1 hour.

BBB properties are induced by astrocytic endfeet (3). Therefore, we have investigated the TfR expression and endocytosis in the absence or presence of astrocytic factors in the cell culture medium. The TfR expression and endocytosis were not changed in the absence or presence of astrocyte-conditioned medium. This is consistent with the "no change" in iron and Tf concentrations in astrocyte-conditioned medium compared to normal cell culture medium. This "no change" was expected, since iron concentrations are the main factor regulating the TfR expression.

For the validation of the TfR for drug targeting, horseradish peroxidase (HRP, 40 kDa) was conjugated to a primary amine group in Tf, in a 1:1 ratio (**chapter 4**). HRP was chosen as a model drug, since it is a well known marker for BBB research (11). The association of the Tf-HRP conjugate by BCEC *in vitro*, was linear up to 10 μg/ml. The time to reach equilibrium at 37 °C was 1 hour, which is similar to the results we obtained with ^{125}I-Tf. Furthermore, with the iron scavenger DFO the association of the Tf-HRP conjugate was increased 2.5 fold.

More importantly, results have shown that both ^{125}I-Tf, as well as Tf-HRP are endocytosed via clathrin-coated vesicles, associated with receptor mediated endocytosis (12, 13). This indicates that the conjugation of a 40 kDa protein drug to a primary amine of Tf, does not modify the mechanism of receptor-ligand internalisation. Therefore, we concluded that the TfR is suitable for drug targeting and delivery to the brain.

3. Liposomes tagged with transferrin for drug delivery to the blood-brain barrier

We have extended our research on drug delivery to the brain from Tf-HRP conjugates to Tf-tagged liposomes. By using a liposomal drug carrier the ratio of the number of drug molecules per targeting vector (i.e. Tf) can be improved. In addition, it is not necessary to chemically modify the drug. Finally, for *in vivo* applications the drug is

protected against degradation in serum. Liposomes themselves are biodegradable, have an acceptable safety profile and their circulation time *in vivo* can be improved by sterically stabilising the liposomes with polyethylene glycol (PEG). Zhang *et al* (2003) have shown that weekly injections of liposomes containing PEG and a protein (monoclonal antibody) did not result in toxicity in rats after 6 weeks.

To be able to couple Tf to the PEG end of the liposomes, PEG with a maleimide group at the distal end of the molecule has been used. Tf is modified at a primary amine group with SATA to insert a thiol group for the irreversible coupling to maleimide. During the preparation of the liposomes it is important to have a high ligand coupling efficiency, as well as to maintain the receptor recognition properties of Tf. For the coupling efficiency the stability of both the thiol group and the maleimide group is important, while for receptor recognition it is important that Tf retains its two iron atoms. Holo-Tf (containing 2 iron atoms) has at least a 1000-fold higher affinity for the TfR than mono-ferric Tf or apo-Tf. In general, EDTA is used for the protection of thiol groups, but EDTA is also a potent metal scavenger. We have optimised the preparation of Tf-tagged liposomes by using TCEP, a thiol protectant which does not scavenge iron from Tf (**chapter 5**).

To determine the association of liposomes by BCEC *in vitro*, HRP was chosen as a liposomal marker (**chapter 6**). HRP is an enzyme, which does not cross the BBB by itself (11); its activity can be readily quantified. Therefore, the activity of the protein drug, rather than the amount of associated drug can be determined. Our data suggest that Tf-tagged liposomes bind to the TfR and are subsequently internalised and routed towards the lysosomes, where HRP is degraded. As a result, we find higher values for the binding (at 4 °C) compared to the association (at 37 °C) of Tf-tagged liposomes. Both the binding and association of Tf-tagged liposomes is consistently higher than of non-tagged liposomes. This indicates that tagging liposomes with Tf does have a beneficial effect for the targeting of liposomes to BCEC *in vitro*. The additional advantage of targeting Tf-tagged liposomes to the TfR is the circumvention of the efflux transporter P-glycoprotein (14, 15). This enables the delivery of drugs to the brain which are substrates for P-glycoprotein, for example anti-tumour drugs or anti-viral drugs.

The Tf-HRP conjugates (**chapter 4**) and the Tf-tagged liposomes (**chapter 6**) are differently endocytosed by BCEC *in vitro*. While our data confirm that Tf-HRP conjugates are endocytosed via clathrin-coated vesicles, the mechanism of endocytosis of

Tf-tagged liposomes is yet unclear. Also, there is a difference in intracellular fate of Tf-HRP conjugates and Tf-tagged liposomes. From literature it is known that non-targeted liposomes are directed towards lysosomes. However, our Tf-tagged liposomes were also directed to the lysosomes. This was not expected since the TfR-Tf complex, is directed towards endosomes upon internalisation. Tf-HRP conjugates, either stay in the endosomes or are released from the endosomes into the cytosol. A fraction of the Tf-HRP conjugates might also be directed to the lysosomes. However, we assume that this is only a small fraction, since the association of Tf-HRP is higher than the binding. The difference in association and intracellular routing between the Tf-HRP conjugates (cytoplasm) and the Tf-tagged liposomes (lysosomes) can be explained by their size (figure 1). Tf has a Stokes-Einstein ratio of approximately 4 nm (16), and HRP of approximately 3 nm (17). In contrast, a liposome is 100 nm in diameter.

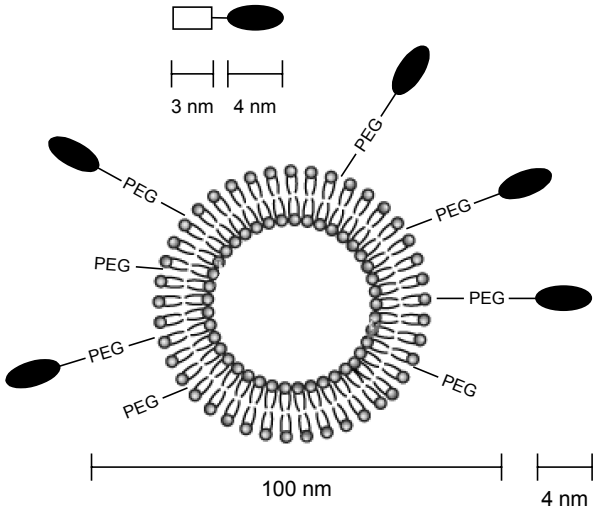


Figure 1: Schematic comparison between HRP encapsulated in a Tf-tagged liposome and a Tf-HRP conjugate. Tf (black oval) is approximately 4 nm (16). HRP (square) has a similar size (approximately 3 nm (17)), while a liposome is 100 nm in diameter.

The difference in size might be the main determinant of the intracellular routing of a particle conjugated to Tf. This information can be used to select either a Tf-drug conjugate or a Tf-tagged liposome, depending on the intracellular target. For example, for the treatment of lysosomal storage disease, it can be useful to target drugs to the

lysosomes, while for other diseases, such as inflammatory disease conditions the cytosol or the nucleus would be more appropriate as a target (18).

3.1 Liposomal drug delivery under inflammatory conditions

Inflammatory disease conditions, such as bacterial meningitis, alter the BBB functioning (19). Lipopolysaccharide (LPS) from gram negative bacteria plays a major role in this altered BBB functioning. Therefore, we have evaluated the TfR expression level, as well as the association after pre-incubation of BCEC with LPS (**chapter 3**). The TfR expression level at 4 °C was not changed after 2 hours pre-incubation with LPS, nor after overnight pre-incubation. However, the cellular protein level after 2 hours incubation with LPS was increased significantly, which gave a biased result when the TfR expression level was expressed per mg cell protein. Since it is known that LPS induces the synthesis of so-called acute phase proteins (20), we have corrected our data for cellular protein levels of the control situation. The association of Tf by the TfR was also not changed after pre-incubation with LPS. These results indicate that the TfR is also suitable for drug targeting and delivery under inflammatory disease conditions.

Targeting liposomes under inflammatory disease conditions was assessed by loading the liposomes with N-acetyl-L-cysteine (NAC). NAC is a hydrophilic free radical scavenger, which can prevent LPS-induced opening of the tight junctions of the BBB *in vitro* (19). In this study, BCEC were co-cultured with rat astrocytes, to form a tight monolayer as measured by TransEndothelial Electrical Resistance (TEER). Overnight pre-incubation with Tf-tagged liposomes loaded with NAC protected the BBB *in vitro* against LPS-induced opening of the tight junctions. However, liposomes that contained just buffer had the same protective effect. Pre-incubation of liposomes with LPS showed a protective effect after at least 6 hours of pre-incubation: the decrease in TEER was less pronounced compared to LPS that was not pre-incubated with liposomes. In addition, pre-incubation of liposomes with fluorescently labelled LPS showed a time-dependent decrease of 15 – 20 % in "free" LPS concentration. In conclusion, it was shown that liposomes have the ability to "scavenge" LPS, thereby decreasing the "free" LPS concentration and thus the LPS effect (**chapter 7**). These results indicate that liposomes are not only suitable as a drug carrier, but also have an intrinsic effect under

inflammatory disease conditions. It needs to be investigated whether the association of LPS by liposomes influences the internalisation of the liposomes.

In addition, NAC might not be the most suitable anti-inflammatory drug, since it is very hydrophilic and can only scavenge one radical per drug molecule. Therefore, more potent scavenger should be considered. Superoxide dismutase (SOD), for example, is an enzyme which can scavenge multiple radicals. Corvo *et al* (2002) have successfully applied liposomes carrying SOD for the treatment of rheumatoid arthritis, a chronic inflammation in joints (21). As an alternative approach, Muzykantov *et al* (1996) have targeted SOD-conjugates to pulmonary endothelium (22). So far, we have used HRP to evaluate drug delivery to the BBB *in vitro*. HRP is not only cheaper, but is also less sensitive to loss of activity. However, the results we have obtained with Tf-HRP conjugates and Tf-tagged liposomes can be extrapolated to SOD. SOD should be delivered to the cytosol, where it can exerts its action. Therefore, Tf-SOD conjugates would be favourable for drug delivery, since these are not primarily directed to the lysosomes. Alternatively, liposomes with endosomal escape possibilities (e.g. with pH dependent fusogenic peptides) can be considered for drug delivery. These liposomes will be discussed in section 4.2.

4. Future perspectives for drug delivery to the brain

The research described in this thesis has been focussing on the use of the TfR for drug delivery to the brain. For this, the endogenous ligand was used, which shows a high affinity for the TfR. The Tf concentration in serum is high, limiting the *in vivo* applicability as a targeting vector. In addition, the TfR is not only expressed at the BBB, but also on hepatocytes, erythrocytes, monocytes and intestinal cells (23). The TfR is therefore not very selective for drug delivery to the brain. However, extensive research by Pardridge *et al* has shown that drug delivery via the TfR is effective (1, 29, 30). Our research was performed *in vitro*, to be able to characterise the TfR and its potential use for drug targeting more mechanistically. It is known that the ideal *in vitro* BBB model to determine endocytosis and transcytosis of compounds and to extrapolate results to the *in vivo* situation does not exist. However, we have used primary cultured bovine BCEC, which are cultured in the presence of astrocyte-conditioned medium. These BCEC are

well characterised for their BBB-like properties, such as the expression of P-glycoprotein, cadherin 5, P-selectin and α_V-integrin, and the activity of γ-GTP enzyme (4). In addition, we have fully characterised the TfR on these BCEC (**chapter 3**). The limitations of an *in vitro* BBB model often involve the paracellular pathway. However, we have focussed on the very first step of drug delivery to the brain: the binding of Tf, Tf-HRP conjugates or Tf-tagged liposomes to the TfR and the subsequent internalisation by the BCEC. Therefore, the primary cultured BCEC were a good model to study the TfR.

4.1. Targeting vectors for brain drug delivery via the transferrin receptor

Our *in vitro* research suggests that the TfR is suitable for drug delivery to the brain. This is in agreement with the *in vivo* research performed by Pardridge and co-workers, who used the monoclonal antibody OX-26 against the rat TfR as a targeting vector. Both Tf and OX-26 have their advantages and disadvantages as a targeting vector. Tf is the endogenous ligand for the TfR, with a relatively high concentration in plasma (22 – 35 μM (23)). Therefore, when using Tf as a targeting vector, competition for the receptor-binding site can be expected. However, our research with Tf-tagged liposomes has shown that liposomes with multiple Tf molecules on their surface, bind to the TfR on BCEC *in vitro* and are internalised in the presence of serum (**chapter 6**). In contrast, OX-26 does not bind to the Tf-binding site and is therefore not displaced by endogenous Tf. However, OX-26 is an antibody against the rat TfR and does not fit into the human TfR. Moreover, rat antibodies will cause immunogenic reactions in humans, unless they are humanised. The preparation of humanised or chimeric antibodies is difficult. In some cases this may lead to a loss of affinity for the target receptor (24).

The TfR is responsible for iron transport to the brain. So far, the intracellular trafficking of Tf and OX-26 upon internalisation via the TfR has not yet been elucidated. Some literature reports suggest transcytosis of Tf across the BCEC, while others claim endocytosis of Tf, followed by an intracellular release of iron and a subsequent return of apo-Tf to the apical side of the BCEC (12, 25, 26). Moos and Morgan (2000) have shown that the transcytosis of iron exceeds the transcytosis of Tf across the BBB, supporting the second theory (27). Furthermore, these authors have

proposed a new theory in which the TfR-Tf complex is transcytosed to the basolateral side of the BCEC, where Tf remains bound to the TfR, but iron is released into the brain extracellular fluid (28). Subsequently apo-Tf bound to the TfR will recycle back to the apical side of the BBB. This theory is supported by data from Zhang and Pardridge (2001) who found a 3.5 fold faster efflux from brain to blood of apo-Tf than holo-Tf (25).

The mechanism of transcytosis of OX-26 is not yet fully elucidated either. Pardridge *et al* have shown efficient drug targeting and delivery to the brain *in vivo* by applying OX-26 (examples can be found in (26, 29-31)). In contrast, Broadwell *et al* (1996) have shown that both Tf and OX-26 are able to cross the BBB, but that the transcytosis of Tf is more efficient (32). Furthermore, Moos and Morgan (2001) have shown that OX-26 mainly accumulates in the BCEC and not in the post-capillary compartment (33). In addition, iron deficiency did not increase OX-26 uptake in rats. Our data as well as literature reports show that iron deficiency causes an increase in TfR expression (**chapter 3**, (6, 34)). Therefore, it is expected that the uptake of OX-26 would increase as well. These data suggest that OX-26 transcytosis might result from a high-affinity accumulation by the BCEC, followed by a non-specific exocytosis at the basolateral side of the BCEC (33). In addition, these authors found a periventricular localisation of OX-26 which suggest that OX-26 probably also is transported across the blood-CSF barrier. Although the mechanism of transcytosis of Tf and OX-26 may not yet be fully elucidated, it is important to realise that drug delivery to the brain via the TfR is efficient.

Preferably, a targeting vector directed to the TfR would be small, non immunogenic and should initialise internalisation of the TfR upon binding. Xu *et al* (2001) have used a single chain antibody Fv fragment against the human TfR, which was tagged with a lipid anchor for insertion into a liposomal bilayer (35). The molecular weight of this antibody fragment, including the lipid anchor was approximately 30 kDa. In addition, Lee *et al* (2001) have used a phage-display technique to find small peptide ligands for the human TfR. They obtained a 7- and a 12-mer peptide, that bind to a different binding site than Tf and are internalised by the TfR. Although these small peptides can also exert immunogenic reactions in humans, they are promising ligands for drug targeting to the TfR on the BBB.

In addition to antibody fragments and peptides, small synthetic ligands can offer great promise for drug delivery to the TfR. These synthetic ligands can either be

developed by molecular modelling of TfR binding sites or by screening of large compound banks. Preferably, a small synthetic ligand should not bind to the Tf-binding site, to avoid competition from endogenous Tf.

4.2 Drug conjugates versus liposomal drug carriers

The research described in this thesis has been focused on the use of Tf-drug conjugates and Tf-tagged liposomes. Drug conjugates are much smaller than liposomes: Tf-HRP conjugates are about 14 times smaller, based on the Stokes-Einstein radii of Tf and HRP (approximately 4 and 3 nm, respectively), than Tf-tagged liposomes (approximately 100 nm in diameter). It is likely that the internalisation of smaller particles by the BCEC is easier and thus quicker. Clathrin-mediated endocytosis, which is associated with the TfR, allows for particles up to 120 nm to be internalised (36). Rejman *et al* (2004) have shown that non-targeted particles of 50 and 100 nm in diameter were internalised by clathrin-mediated endocytosis, and with similar kinetics (37). Particles which were 200 nm in diameter were also internalised via clathrin-mediated endocytosis, but their uptake was slower. The amount of 100 and 200 nm particles that were internalised was respectively 3 – 4 and 8 – 10 times lower compared to the uptake of 50 nm particles (37). Although the uptake of larger particles is lower, these particles can encapsulate more drug molecules, resulting in a similar intracellular drug concentration.

Besides internalisation, intracellular trafficking also seems to be different for Tf-drug conjugates and Tf-tagged liposomes. Tf-tagged liposomes are directed towards the lysosomes, while Tf-HRP conjugates stay in the endosomes or are released into the cytosol. Beyer *et al* (2001) have found similar results for doxorubicin: liposomal doxorubicin showed less endocytosis than free doxorubicin, but the intracellular distribution of liposomal doxorubicin was similar to free doxorubicin (38). In contrast, Tf- and BSA-doxorubicin conjugates were differently distributed. These findings are similar to our results and could imply that depending on the intracellular target, a Tf-drug conjugate or a Tf-tagged liposome is preferred.

The Tf-tagged liposomes we have used were made of conventional lipids (egg phosphatidylcholine and cholesterol) and were sterically stabilised with PEG. Over the

years new techniques have been developed to prevent the liposomal drug from degradation by the lysosomes. First, pH sensitive liposomes have been developed, which dissociate at low pH, releasing their contents in the endosomes. Secondly, in combination with a fusogenic peptide, such as GALA or TAT (39, 40), the endosomal membrane is disrupted and the drug is released into the cytosol. Recently, Kakudo *et al* (2004) have demonstrated that Tf-modified liposomes equipped with a pH sensitive fusogenic peptide efficiently fuse with the endosomal membrane (41). This drug delivery system allows for the efficient delivery of gene therapeutics to the brain.

It depends on the characteristics of the drug, as well as on the intracellular target whether a drug conjugate or a liposomal drug carrier is the best option for the delivery of the drug to the target site. If the drug is a hydrophilic protein which is rather large, the encapsulation efficiency by a small liposome is not as high as it is for smaller drug molecules. On the other hand, for the incorporation of a drug into a liposome it is not necessary to chemically modify the drug. Furthermore, the drug is protected against degradation in serum. For gene therapeutics, stability in serum is an important issue, since nucleic acids are susceptible to ribonuclease degradation. Therefore, liposomal drug carriers are more suitable for gene therapeutics compared to drug conjugates. Recently, interference RNA (RNAi) has shown great promise for silencing gene expression at the post-transcriptional level (42, 43). Zhang *et al* (2004) used RNAi to silence oncogenic genes in solid cancers, by using liposomes tagged with antibodies against the TfR, as well as against the insulin receptor (44).

In summary, it depends on the drug characteristics, as well as on the intracellular target, whether a drug conjugate or a liposomal drug carrier is preferred for the selective delivery to the BBB and the brain.

4.3. Brain drug delivery: a selection of targets

The TfR is suitable for drug targeting to the brain, since it is abundantly expressed at the BBB and is actively endocytosing its ligand. However, by long-term use of the TfR for drug targeting the uptake of Tf might be disturbed and this may affect the intracellular iron homeostasis. Iron plays a role in metabolic processes and acts as co-factor for several enzymes. Long-term iron deficiency in the brain of children has been

shown to lead to impaired cognition and abnormal behaviour (23). In contrast, an excess of iron may lead to the formation of free radicals and tissue damage (23). This may be a contributing factor in the development of several neurological diseases (Baker & Morgan 1994), such as Alzheimer's or Parkinson's disease. However, the BBB is a dynamic organ, which tightly regulates the brain homeostasis. Furthermore, the large intracellular pool of TfR (90 % of the total TfR expression, **chapter 3**) and the efficient transcriptional control of the TfR, as well as ferritin (7, 8) suggest that iron sequestration by cells or organs is strictly regulated. Therefore, the iron homeostasis might not be influenced by targeting the TfR, but it may cause a receptor downregulation and thus a lower targeting efficiency.

Other physiological drug targeting and delivery strategies that are under investigation involve adsorptive-mediated transcytosis, carrier-mediated transcytosis or receptor-mediated transcytosis. Zhang *et al* (2004) have used liposomes tagged with two antibodies: one against the TfR, and the other against the insulin receptor (44, 45). This double labelling enables the liposomes to cross the BBB as well as the cell- and the nuclear membrane.

Recently, new techniques, such as gene array technologies and subtractive antibody expression cloning have been developed which may lead to new targets at the BBB (46-49). Shusta *et al* have shown a high and specific expression of the Lutheran membrane glycoprotein and the membrane cofactor protein CD46 in BBB endothelial cells compared to other organs, such as kidney and liver (50, 51). These techniques could also be used to find targets for drug delivery which are up-regulated in a disease state (e.g. under inflammatory disease conditions).

4.4 Learning from pathogens for brain drug delivery

Crossing the BBB via endogenous, physiological transport systems might not be the only attractive strategy for drug delivery to the brain. Viral, bacterial, fungal and parasitic pathogens are able to cross the BBB and infect the brain. The limited number of pathogens crossing the BBB suggests that these have very specific attributes (52). Understanding the mechanisms by which these pathogens cross the BBB might not only help to develop new treatments against infections, but can also help to develop new

targeting strategies for drug delivery to the brain for the treatment of other diseases. Bacteria, which replicate intracellularly are likely to use infected leukocytes as a 'Trojan horse' to cross the BBB, while it is yet unknown how extracellularly replicating bacteria cross the BBB (52).

Another example of a pathogen crossing the BBB, is a parasite, *Toxoplasma gondii*, which can not only cross the intestinal barrier, but also the placental barrier, the blood-retina barrier and the BBB (53). It is yet unknown how this parasite is able to cross these barriers, although some evidence suggests that there are analogies to leukocyte trafficking. Leukocyte trafficking across the BBB is caused by a cascade of effects. The first step is the recognition of adhesion molecules on the surface of leukocytes by their corresponding ligands on the BCEC, after which they are to cross the BBB paracellularly (54). Parasites could use a similar mechanism of paracellular transport, after recognition and possible activation of a cellular marker.

Pathogens can also bypass the BBB to reach the brain (52, 55). This can be achieved by using retrograde axonal transport through sensory or motor fibers. Prion disease is an example of a pathogen that bypasses the BBB in this way. Recently, Haik *et al* (2004) have reported that prions probably use the sympathetic noradrenergic neurons to reach the brain (55).

5. Conclusion

The research described in this thesis was initiated to meet the challenge of drug delivery across the BBB. The focus of this thesis was to explore the possibilities for drug delivery to the brain via the TfR. Although drug delivery to the brain via the TfR is not selective, it is effective. We have chosen for a mechanistic approach in which the binding and association of Tf, Tf-drug conjugates and Tf-tagged liposomes by BCEC *in vitro* was determined. The efficiency of delivery depends mainly on the size of the cargo, a 40 kDa protein does not affect the association of Tf by the TfR, while a liposomal drug carrier (100 nm in diameter) affects both the association and the intracellular routing.

In conclusion, results obtained in our *in vitro* BBB model have shown that the TfR is an attractive target for the delivery of drugs to the brain.

6. References

1. Pardridge, W. M. (2001) Drug targeting, drug discovery, and brain drug development. In *Brain drug targeting - The future of brain drug development* (W. M. Pardridge, ed.), University Press, Cambridge, pp. 1-12
2. Pardridge, W. M. (1991) Overview of Blood-Brain Barrier Transport Biology and Experimental Methodologies. In *Peptide Drug Delivery to the Brain* (W. M. Pardridge, ed.), Raven Press, New York, pp. 52 - 98
3. Rubin, L. L. and Staddon, J. M. (1999) The cell biology of the blood-brain barrier. *Annu Rev Neurosci* **22** 11-28
4. Gaillard, P. J., Voorwinden, L. H., Nielsen, J. L., Ivanov, A., Atsumi, R., Engman, H., Ringbom, C., de Boer, A. G. and Breimer, D. D. (2001) Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur J Pharm Sci* **12** (3): 215-222
5. Bridges, K. R. and Cudkowicz, A. (1984) Effect of iron chelators on the transferrin receptor in K562 cells. *J Biol Chem* **259** (21): 12970-12977
6. van Gelder, W., Huijskes-Heins, M. I., Cleton-Soeteman, M. I., van Dijk, J. P. and van Eijk, H. G. (1998) Iron uptake in blood-brain barrier endothelial cells cultured in iron-depleted and iron-enriched media. *J Neurochem* **71** (3): 1134-1140
7. Kuhn, L. C. (1991) mRNA-protein interactions regulate critical pathways in cellular iron metabolism. *Br J Haematol* **79** (1): 1-5
8. Casey, J. L., Koeller, D. M., Ramin, V. C., Klausner, R. D. and Harford, J. B. (1989) Iron regulation of transferrin receptor mRNA levels requires iron- responsive elements and a rapid turnover determinant in the 3' untranslated region of the mRNA. *Embo J* **8** (12): 3693-3699.
9. Britton, R. S., Leicester, K. L. and Bacon, B. R. (2002) Iron toxicity and chelation therapy. *Int J Hematol* **76** (3): 219-228
10. Dayani, P. N., Bishop, M. C., Black, K. and Zeltzer, P. M. (2004) Desferoxamine (DFO)--mediated iron chelation: rationale for a novel approach to therapy for brain cancer. *J Neurooncol* **67** (3): 367-377
11. Banks, W. A. and Broadwell, R. D. (1994) Blood to brain and brain to blood passage of native horseradish peroxidase, wheat germ agglutinin, and albumin: pharmacokinetic and morphological assessments. *J Neurochem* **62** (6): 2404-2419
12. Pardridge, W. M., Eisenberg, J. and Yang, J. (1987) Human blood-brain barrier transferrin receptor. *Metabolism* **36** (9): 892-895
13. Nguyen, H. M., Cahill, C. M., McPherson, P. S. and Beaudet, A. (2002) Receptor-mediated internalization of [3H]-neurotensin in synaptosomal preparations from rat neostriatum. *Neuropharmacology* **42** (8): 1089-1098
14. Huwyler, J., Cerletti, A., Fricker, G., Eberle, A. N. and Drewe, J. (2002) By-passing of P-glycoprotein using immunoliposomes. *J Drug Target* **10** (1): 73-79

15. Fricker, G. and Miller, D. S. (2004) Modulation of drug transporters at the blood-brain barrier. *Pharmacology* **70** (4): 169-176
16. Enns, C. A. and Sussman, H. H. (1981) Physical characterization of the transferrin receptor in human placentae. *J Biol Chem* **256** (19): 9820-9823
17. Ojteg, G., Nygren, K. and Wolgast, M. (1987) Permeability of renal capillaries. I. Preparation of neutral and charged protein probes. *Acta Physiol Scand* **129** (3): 277-286
18. Everts, M., Schraa, A. J., de Leij, L. F. M. H., Meijer, D. K. F. and Molema, G. (2001) Vascular endothelium in inflamed tissue as a target for site selective delivery of drugs. In *Drug Targeting, Organ-Specific Strategies* (D. K. F. Meijer and G. Molema, eds), Wiley-VCH, Weinheim, pp. 171-197
19. Gaillard, P. J., de Boer, A. B. and Breimer, D. D. (2003) Pharmacological investigations on lipopolysaccharide-induced permeability changes in the blood-brain barrier in vitro. *Microvasc Res* **65** (1): 24-31
20. Baumann, H. and Gauldie, J. (1994) The acute phase response. *Immunol Today* **15** (2): 74-80
21. Corvo, L. M., Jorge, J. C., van't Hof, R., Cruz, M. E., Crommelin, D. J. and Storm, G. (2002) Superoxide dismutase entrapped in long-circulating liposomes: formulation design and therapeutic activity in rat adjuvant arthritis. *Biochim Biophys Acta* **1564** (1): 227-236
22. Muzykantov, V. R., Atochina, E. N., Ischiropoulos, H., Danilov, S. M. and Fisher, A. B. (1996) Immunotargeting of antioxidant enzyme to the pulmonary endothelium. *Proc Natl Acad Sci U S A* **93** (11): 5213-5218
23. Baker, E. and Morgan, E. H. (1994) Iron transport. In *Iron metabolism in health and disease* (Brock, ed.), Saunders, London, pp. 63-95
24. Pardridge, W. M. (2001) Vector discovery: genetically engineered Trojan horses for drug targeting. In *Brain drug targeting - The future of brain drug development* (W. M. Pardridge, ed.), University Press, Cambridge, pp. 126-154
25. Zhang, Y. and Pardridge, W. M. (2001) Rapid transferrin efflux from brain to blood across the blood-brain barrier. *J Neurochem* **76** (5): 1597-1600
26. Pardridge, W. M., Buciak, J. L. and Friden, P. M. (1991) Selective transport of an anti-transferrin receptor antibody through the blood-brain barrier in vivo. *J Pharmacol Exp Ther* **259** (1): 66-70
27. Moos, T. and Morgan, E. H. (2000) Transferrin and transferrin receptor function in brain barrier systems. *Cell Mol Neurobiol* **20** (1): 77-95
28. Moos, T. and Morgan, E. H. (2004) The significance of the mutated divalent metal transporter (DMT1) on iron transport into the Belgrade rat brain. *J Neurochem* **88** (1): 233-245
29. Pardridge, W. M., Boado, R. J. and Kang, Y. S. (1995) Vector-mediated delivery of a polyamide ("peptide") nucleic acid analogue through the blood-brain barrier in vivo. *Proc Natl Acad Sci U S A* **92** (12): 5592-5596
30. Shi, N., Boado, R. J. and Pardridge, W. M. (2001) Receptor-mediated gene targeting to tissues in vivo following intravenous administration of pegylated immunoliposomes. *Pharm Res* **18** (8): 1091-1095
31. Zhang, Y. F., Boado, R. J. and Pardridge, W. M. (2003) Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. *Pharm Res* **20** (11): 1779-1785
32. Broadwell, R. D., Baker-Cairns, B. J., Friden, P. M., Oliver, C. and Villegas, J. C. (1996) Transcytosis of protein through the mammalian cerebral epithelium and endothelium. III. Receptor-mediated

transcytosis through the blood-brain barrier of blood-borne transferrin and antibody against the transferrin receptor. *Exp Neurol* **142** (1): 47-65

33. Moos, T. and Morgan, E. H. (2001) Restricted transport of anti-transferrin receptor antibody (OX26) through the blood-brain barrier in the rat. *J Neurochem* **79** (1): 119-129
34. Morgan, E. H. (1996) Iron metabolism and transport. In *Hepatology. A textbook of liver disease*, Vol. 1 (D. Zakin and T. D. Boyer, eds), Saunders, Philadelphia, pp. 526-554
35. Xu, L., Tang, W. H., Huang, C. C., Alexander, W., Xiang, L. M., Pirollo, K. F., Rait, A. and Chang, E. H. (2001) Systemic p53 gene therapy of cancer with immunolipoplexes targeted by anti-transferrin receptor scFv. *Mol Med* **7** (10): 723-734
36. Conner, S. D. and Schmid, S. L. (2003) Regulated portals of entry into the cell. *Nature* **422** (6927): 37-44
37. Rejman, J., Oberle, V., Zuhorn, I. S. and Hoekstra, D. (2004) Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J* **377** (Pt 1): 159-169
38. Beyer, U., Rothern-Rutishauser, B., Unger, C., Wunderli-Allenspach, H. and Kratz, F. (2001) Differences in the intracellular distribution of acid-sensitive doxorubicin-protein conjugates in comparison to free and liposomal formulated doxorubicin as shown by confocal microscopy. *Pharm Res* **18** (1): 29-38
39. Parente, R. A., Nir, S. and Szoka, F. C., Jr. (1990) Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry* **29** (37): 8720-8728
40. Torchilin, V. P. and Levchenko, T. S. (2003) TAT-liposomes: a novel intracellular drug carrier. *Curr Protein Pept Sci* **4** (2): 133-140
41. Kakudo, T., Chaki, S., Futaki, S., Nakase, I., Akaji, K., Kawakami, T., Maruyama, K., Kamiya, H. and Harashima, H. (2004) Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system. *Biochemistry* **43** (19): 5618-5628
42. Shi, F. (2004) *On the mechanism of cationic lipid-mediated delivery of oligonucleotides: towards and antisense therapy (Thesis, University of Groningen, the Netherlands).*
43. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391** (6669): 806-811
44. Zhang, Y., Zhang, Y. F., Bryant, J., Charles, A., Boado, R. J. and Pardridge, W. M. (2004) Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clin Cancer Res* **10** (11): 3667-3677
45. Zhang, Y., Zhu, C. and Pardridge, W. M. (2002) Antisense gene therapy of brain cancer with an artificial virus gene delivery system. *Mol Ther* **6** (1): 67-72
46. Pardridge, W. M. (2003) Blood-brain barrier genomics and the use of endogenous transporters to cause drug penetration into the brain. *Curr Opin Drug Discov Devel* **6** (5): 683-691
47. Kallmann, B. A., Wagner, S., Hummel, V., Buttmann, M., Bayas, A., Tonn, J. C. and Rieckmann, P. (2002) Characteristic gene expression profile of primary human cerebral endothelial cells. *Faseb J* **16** (6): 589-591

48. Marroni, M., Kight, K. M., Hossain, M., Cucullo, L., Desai, S. Y. and Janigro, D. (2003) Dynamic in vitro model of the blood-brain barrier. Gene profiling using cDNA microarray analysis. *Methods Mol Med* **89** 419-434
49. Shusta, E. V., Boado, R. J., Mathern, G. W. and Pardridge, W. M. (2002) Vascular genomics of the human brain. *J Cereb Blood Flow Metab* **22** (3): 245-252
50. Shusta, E. V., Boado, R. J. and Pardridge, W. M. (2002) Vascular proteomics and subtractive antibody expression cloning. *Mol Cell Proteomics* **1** (1): 75-82
51. Shusta, E. V., Zhu, C., Boado, R. J. and Pardridge, W. M. (2002) Subtractive expression cloning reveals high expression of CD46 at the blood-brain barrier. *J Neuropathol Exp Neurol* **61** (7): 597-604
52. Nassif, X., Bourdoulous, S., Eugene, E. and Couraud, P. O. (2002) How do extracellular pathogens cross the blood-brain barrier? *Trends Microbiol* **10** (5): 227-232
53. Barragan, A. and Sibley, L. D. (2003) Migration of Toxoplasma gondii across biological barriers. *Trends Microbiol* **11** (9): 426-430
54. Brown, K. A. (2001) Factors modifying the migration of lymphocytes across the blood-brain barrier. *Int Immunopharmacol* **1** (12): 2043-2062
55. Haik, S., Faucheux, B. A. and Hauw, J. J. (2004) Brain targeting through the autonomous nervous system: lessons from prion diseases. *Trends Mol Med* **10** (3): 107-112

Chapter 9

Nederlandse samenvatting

Synopsis in Dutch

De transferrine receptor op de bloed-hersenbarrière:

Onderzoek naar de mogelijkheden voor selectief transport van geneesmiddelen naar de hersenen

Inhoud hoofdstuk 9

1. Achtergrond en doel van het beschreven onderzoek

Vele ziekten in de hersenen, zoals bijvoorbeeld de ziekte van Alzheimer, de ziekte van Parkinson, depressie, schizofrenie en multiple sclerose, kunnen nog niet behandeld worden. Dit heeft onder meer te maken met de aanwezigheid van de bloed-hersenbarrière (BHB). De BHB bevindt zich tussen het bloed en de hersenen en beschermt en voedt de hersenen. Dit wordt bereikt door selectief transport van voedingsmiddelen, zoals glucose, zuurstof en aminozuren naar de hersenen, en het tegenhouden en uitscheiden van lichaamsvreemde of toxische stoffen. In het algemeen kan gezegd worden dat alleen kleine vetoplosbare stoffen de BHB goed kunnen passeren. Echter, voor de meeste geneesmiddelen geldt dat ze de BHB met moeite passeren.

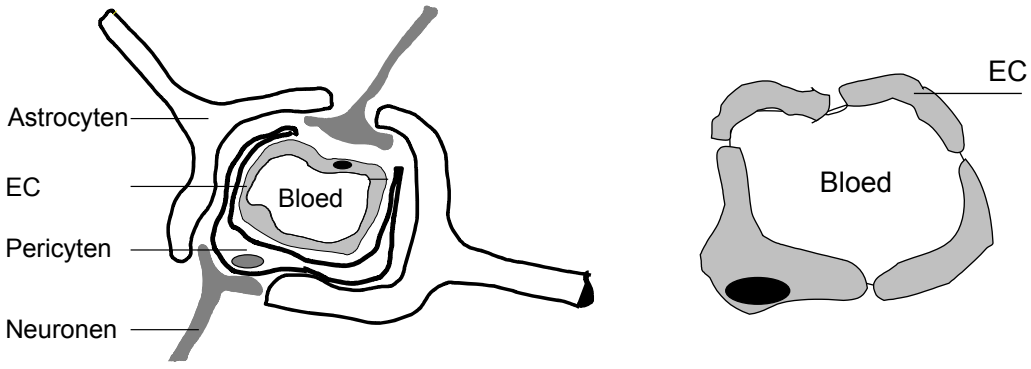
Het onderzoek zoals beschreven in dit proefschrift was gericht op de transferrine receptor voor het transport van stoffen over de BHB en werd volledig uitgevoerd *in vitro*. Hiervoor werd gebruik gemaakt van endotheelcellen, geïsoleerd uit de kleinste bloedvaten (capillairen) van kalfshersenen. De transferrine receptor is verantwoordelijk voor de ijzeropname door de hersenen. Het ijzer wordt vervoerd door transferrine, het endogene ligand voor de transferrine receptor. In een drietal stappen is onderzocht of de transferrine receptor geschikt is voor geneesmiddeltransport naar de hersenen. Voor de eerste stap is gebruik gemaakt van radioactief gelabeld transferrine, om de aanwezigheid van de receptor op het *in vitro* BHB model aan te tonen. Vervolgens is een enzym (peroxidase uit mierikswortel) aan transferrine gekoppeld, om vast te stellen of de transferrine receptor geschikt is om een lading (cargo) naar de hersenen te transporteren. Als laatste stap is voor een grotere lading gekozen, namelijk een liposoom beladen met transferrine. Liposomen zijn vetbolletjes, waarin geneesmiddelen verpakt kunnen worden. Deze liposomen kunnen langer in het bloed blijven circuleren en kunnen selectief naar een bepaald orgaan gestuurd worden (bijvoorbeeld door koppeling aan transferrine worden liposomen naar de hersenen gericht).

In de volgende paragrafen zal nader worden ingegaan op de biologische aspecten en de functie van de bloed-hersenbarrière, de transferrine receptor en het liposomale geneesmiddeltransport.

2. De bloed-hersenbarrière: biologie en functie

Het bestaan van de bloed-hersenbarrière (BHB) is aan het eind van de 19e eeuw ontdekt door Ehrlich en Goldman. Ehrlich injecteerde een blauwe kleurstof in de bloedbaan van een rat en vond dat deze stof niet in de hersenen kwam. Zijn opvolger Goldman heeft vervolgens de blauwe kleurstof in de hersenen geïnjecteerd. In deze situatie werden alleen de hersenen en de ruggengraat blauw gekleurd. Deze bevindingen hebben aanleiding gegeven tot het formuleren van het concept van de BHB. Sindsdien is er veel onderzoek gedaan naar de morfologie, biologie en functie van de BHB.

De BHB wordt gevormd door een viertal celtypen (figuur 1). De belangrijkste barrière wordt gevormd door de capillaire endotheelcellen, die de wand van de kleinste bloedvaten in de hersenen bekleden. Deze endotheelcellen liggen zeer dicht tegen elkaar aan, waardoor er alleen kleine openingen (zogenaamde 'tight junctions') aanwezig zijn tussen de cellen. In andere delen van het lichaam liggen de endotheelcellen verder uit elkaar, waardoor stoffen uit het bloed "langs" de cellen kunnen diffunderen. Een ander kenmerk van de endotheelcellen in de hersencapillairen is de geringe permeabiliteit van de cellen zelf. Bovendien komt P-glycoproteïne, een eiwit dat actief stoffen uit de cellen verwijdert, in hoge mate voor op het oppervlak van BHB endotheelcellen. Andere celtypen die betrokken zijn bij de barrière-functie zijn de astrocyten, die met hun uiteinde de capillairen omgeven en zorgen voor de specifieke BHB eigenschappen van de endotheelcellen. Ook andere hersencellen, zoals neuronen en pericyten zijn betrokken bij de BHB.



Figuur 1: Schematische vergelijking tussen een hersencapillair (links) en een capillair uit een ander deel van het lichaam (rechts). De BHB wordt gevormd door endotheelcellen (EC) die dicht tegen elkaar aanliggen en omgeven zijn door astrocyten, pericyten en neuronen. Endotheelcellen uit de rest van het lichaam liggen minder dicht tegen elkaar aan en zijn daardoor beter doorlaatbaar.

Naast de BHB zijn er ook barrières te vinden tussen de hersenen en de vloeistof uit de hersenen (liquor) en tussen het bloed en de hersenvloeistof. De BHB wordt echter gezien als de belangrijkste barrière voor stoffen om de hersenen te bereiken, omdat het met een oppervlak van ca. 20 m^2 en een lengte van ca. 650 km de grootste barrière in de hersenen vormt.

Voor het onderzoek, beschreven in dit proefschrift is gebruik gemaakt van een zogenaamd *in vitro* BHB model. Hiervoor worden de kleinste capillairen uit kalfshersenen geïsoleerd. Deze capillairen worden vervolgens in celkweekmedium bij 37 ºC gekweekt, totdat er na ca. 5 dagen genoeg endotheelcellen uitgegroeid zijn. Deze endotheelcellen worden overgebracht naar een plaat met kleine individuele kweekbakjes, waarna er na nogmaals ca. 5 dagen een experiment kan worden uitgevoerd. Tevens worden uit de hersenen van jonge ratten hersencellen, met name astrocyten, geïsoleerd. Deze astrocyten worden in kweekflessen gedurende enkele weken gekweekt. Het celkweekmedium van deze astrocyten wordt bewaard omdat hier stoffen inzitten die door de astrocyten zijn uitgescheiden. Dit zogenaamd astrocyt-geconditioneerd medium wordt toegevoegd aan de capillairen en aan de endotheelcellen, zodat de specifieke eigenschappen van de BHB geïnduceerd worden. Voor de experimenten beschreven in **hoofdstuk 7**, werd zelfs gebruik gemaakt van endotheelcellen die in direct contact staan met astrocyten. Hiervoor worden er op een doorlaatbaar kunststof membraan, astrocyten aan de onderkant gekweekt en endotheelcellen aan de bovenkant. Daardoor ontstaan twee aparte compartimenten, die representatief zijn voor de "bloed-zijde" en de "hersen-zijde" van de BHB.

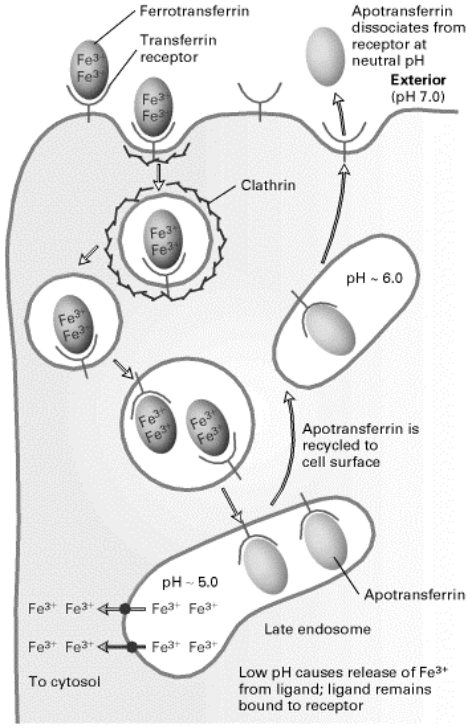
De functie van de BHB is het behouden van homeostase (evenwicht) in de hersenen. Dit wordt bereikt door het selectief doorlaten van voedingsstoffen, terwijl de meeste lichaamsvreemde stoffen worden tegengehouden. Voedingstoffen, zoals bijvoorbeeld glucose, zuurstof, ijzer en aminozuren kunnen via een aantal specifieke transportmechanismen op de BHB de hersenen bereiken. Grofweg kan onderscheid gemaakt worden tussen passief transport (alleen mogelijk voor kleine, vetoplosbare stoffen) en actief transport (d.w.z. dat het transport energie kost).

Een aantal van de beschikbare transportmechanismen wordt ook gebruikt voor geneesmiddeltransport naar de hersenen. Het meest bekende voorbeeld hiervan is L-dopa®, een geneesmiddel dat door een transportmechanisme voor aminozuren naar de hersenen wordt gebracht, waarna het in de hersenen wordt omgezet in dopamine. Als

gevolg van de ziekte van Parkinson neemt de concentratie dopamine in de hersenen af, maar kan dus op deze manier worden aangevuld. De symptomen van de ziekte van Parkinson worden hiermee bestreden. Ook andere transport mechanismen worden gebruikt of onderzocht voor geneesmiddeltransport naar de hersenen, zoals bijvoorbeeld de insuline receptor en de hexose tranporter (transport van glucose). Ook wordt het gebruik van positief geladen albumine (een eiwit wat normaal niet geladen is en in hoge concentraties in het bloed voorkomt) of melano-transferrine (wat niet via de transferrine receptor wordt opgenomen) onderzocht voor geneesmiddeltransport naar de hersenen.

3. De transferrine receptor: mogelijkheden voor geneesmiddeltransport naar de hersenen

Het onderzoek beschreven in dit proefschrift was gericht op de transferrine receptor (TfR). Deze receptor is verantwoordelijk voor het ijzertransport naar de hersenen. Ook komt deze receptor voor op rode bloedcellen, levercellen en cellen van de darmen. Figuur 2 laat schematisch zien hoe de opname van ijzer door endotheelcellen van de BHB verloopt. De TfR heeft bij een fysiologische zuurgraad (pH) een hoge affiniteit voor transferrine (Tf) dat 2 ijzeratomen bevat. Na binding van Tf aan de receptor wordt het TfR-Tf complex door de cel geïnternaliseerd in zogenaamde blaasjes, die gecoat zijn met het eiwit clathrine. Vervolgens wordt, nadat het clathrine losgelaten heeft, de zuurgraad in deze blaasjes (endosomen) verhoogd waardoor ijzer loslaat uit het TfR-Tf complex en via een transporteiwit voor metalen naar het cytosol (cellulaire vloeistof) wordt getransporteerd. Hier wordt het ijzer opgeslagen of verder getransporteerd naar de hersenen. Het TfR-Tf complex wordt na afgifte van ijzer terug getransporteerd naar het bloed, waar Tf loslaat van de receptor en weer opnieuw twee ijzeratomen kan opnemen.



Figuur 2: Schematisch overzicht van de opname van Tf door endotheelcellen van de BHB. Tf beladen met twee ijzeratomen (ferrotransferrin) bindt aan de receptor en wordt vervolgens opgenomen in clathrine-gecoate blaasjes. Nadat clathrine losgelaten heeft, wordt de pH in de blaasjes (endosomen) verlaagd en kan ijzer loslaten. Tf zonder ijzer (apotransferrin) wordt vervolgens met de TfR terug gebracht naar het oppervlak van de cel, terwijl het ijzer uit de endosomen wordt getransporteerd.

In **hoofdstuk 3** is met behulp van radioactief gelabeld Tf (^{125}I-Tf) de receptor dichtheid op de endotheelcellen van het *in vitro* BHB model bepaald. Tevens is de mate en de route van opname, alsook de invloed van de ijzerconcentratie in het celkweek medium hierop bestudeerd. In tabel 1 zijn de maximale TfR dichtheden bij 4 °C (temperatuur waarbij de TfR niet internaliseert) en bij 37 °C weergegeven. Ook is bij 4 °C een onderscheid gemaakt tussen de totale dichtheid van de TfR en de dichtheid van de TfR op het oppervlak van de endotheel cellen van de BHB. Hieruit bleek dat maar ca. 10 % van de totale TfR dichtheid op het oppervlak tot expressie wordt gebracht.

In de situatie waarbij ijzer wordt weggevangen door een ijzer-chelator (deferoxamine mesylate, DFO) wordt de TfR expressie groter, vooral op het oppervlak. Dit houdt in dat er niet alleen nieuwe TfR worden aangemaakt, maar ook dat er een verschuiving in expressie plaats vindt ten gunste van het celoppervlak. Als er extra ijzer aanwezig is, door toevoeging van $FeCl_3$ wordt de TfR minder tot expressie gebracht. Deze

bevindingen komen overeen met eerdere onderzoeken waaruit bleek dat ijzer de belangrijkste factor is in de regulatie van de TfR expressie. Ook heeft ons onderzoek bevestigd dat op de endotheelcellen van de BHB de TfR via clathrine-gecoate blaasjes wordt opgenomen. De opname is ook zeer effectief; na 1 uur incubatie van de endotheelcellen met radioactief gelabeld Tf werd ruim 70% in de cellen aangetroffen.

	maximale receptor dichtheid (fmol/µg cel eiwit)		
	4 °C		37 °C
	totale TfR expressie	extracellulaire TfR expressie	
controle	1.37	0.13	0.90
(BI)	(1.15 – 1.59)	(0.10 – 0.16)	(0.78 – 1.01)
DFO	3.68	0.63	2.31
(BI)	(2.73 – 4.63)	(0.55 – 0.71)	(2.03 – 2.59)
$FeCl_3$	1.64	niet detecteerbaar	0.56
(BI)	(1.09 – 2.19)		(0.49 – 0.63)

Tabel 1: TfR expressie dichtheden in normale situatie (controle), met ijzer chelator (DFO) en met extra ijzer ($FeCl_3$). De weergegeven waarden zijn gemiddelde waarden uit minimaal 3 experimenten, uitgevoerd in triplo, tussen haakjes staat het betrouwbaarheidsinterval (BI) vermeld.

Vervolgens is de invloed van astrocyt-geconditioneerd medium bepaald, omdat bekend is dat astrocyten BHB eigenschappen induceren in endotheelcellen. De expressie van de TfR bleef echter onveranderd in de aan- of afwezigheid van astrocyt-geconditioneerd medium. Dit werd bevestigd door de onveranderde Tf en ijzerconcentraties in astrocyt-geconditioneerd medium in vergelijking met gewoon celkweek medium.

Uit de resultaten in **hoofdstuk 3** kan worden geconcludeerd dat de TfR aanwezig is op de endotheelcellen van de BHB en actief Tf internaliseert. Ook is aangetoond dat de TfR expressie in belangrijke mate afhangt van de ijzerconcentratie.

In **hoofdstuk 4** is beschreven dat Tf in staat is een enzym (peroxidase, geïsoleerd uit mierikswortel) naar de BHB te sturen. Dit enzym wordt veelvuldig gebruikt in

onderzoeken naar de BHB, omdat het de BHB niet kan passeren. Ook wordt het, als het toch in de endotheelcellen komt, afgebroken. Een bijkomend voordeel is de eenvoudige methode waarmee de activiteit van dit enzym (omzetting van water en zuurstof naar peroxide) bepaald kan worden. Door Tf aan het enzym te koppelen werd dit enzym wel door de cellen opgenomen.

Tenslotte heeft ons onderzoek aangetoond dat het Tf-enzym conjugaat specifiek door de TfR wordt opgenomen via clathrine-gecoate blaasjes. Ook werd de opname verhoogd door de ijzerchelator DFO te gebruiken. Dit houdt in dat Tf waar iets aan gekoppeld is, ook via de TfR kan worden opgenomen. De belangrijkste conclusie was dan ook dat de TfR in principe geschikt is voor de selectieve versturing van geneesmiddelen naar de hersenen.

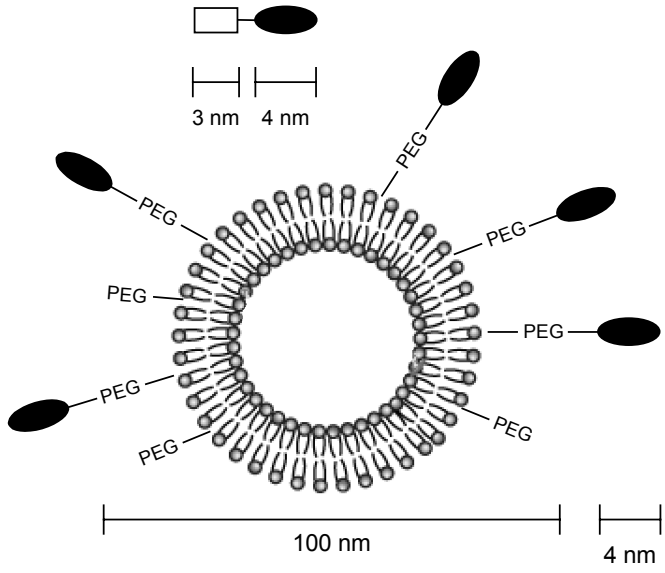
4. Geneesmiddeltransport met behulp van transferrine gelabelde liposomen

Nadat aangetoond was dat de TfR geschikt was voor transport van geneesmiddelen naar de hersenen, werd het onderzoek uitgebreid door liposomen te gebruiken. In liposomen kan namelijk een variëteit aan geneesmiddelen worden vervoerd. Bovendien veroorzaken ze geen immunologische reacties en zijn ze biologisch afbreekbaar. In eerste instantie werd, zoals beschreven in **hoofdstuk 5**, de bereiding van liposomen met Tf op het oppervlak geoptimaliseerd. Met behulp van een chemische reactie werd, in 2 stappen, een thiolaat bevattende groep in Tf aangebracht. In een volgende stap kon deze thiolaat dan reageren met een maleimide groep op het oppervlak van de liposomen om zo een stabiele verbinding te vormen. Hierbij was het enerzijds belangrijk om de thiolaat te stabiliseren, zodat de koppeling met maleimide plaats kon vinden. Anderzijds was het ook zeer belangrijk dat de ijzeratomen in Tf niet weggevangen werden, omdat Tf met twee ijzeratomen de hoogste affiniteit heeft voor de TfR. Hiervoor is uiteindelijk een chemische stof gevonden, te weten Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), die de thiolaat stabiliseert zonder ijzeratomen weg te vangen.

Vervolgens zijn, in **hoofdstuk 6**, deze Tf-gelabelde liposomen met het peroxidase enzym uit mierikswortel beladen, waarna de opname van deze liposomen door endotheelcellen is bepaald. Bij het bereiden van deze liposomen is ervoor gekozen om de deeltjesgrootte maximaal ca. 100 nm te maken, omdat opname van stoffen door

clathrine-gecoate blaasjes beperkt is tot een grootte van ca. 120 nm. Uit dit onderzoek bleek herhaaldelijk dat de binding van de liposomen aan de cellen hoger was dan de opname door de cellen. Hierbij moet wel in gedachten gehouden worden dat de activiteit van het enzym bepaald wordt en niet de hoeveelheid. Ons onderzoek heeft aangetoond dat de liposomen zeer waarschijnlijk wel door de endotheelcellen worden opgenomen, maar dat deze vervolgens intracellulair naar de lysosomen worden gestuurd. In deze lysosomen vindt afbraak plaats van zowel de liposomen als hun inhoud. Hierdoor is het niet meer mogelijk om het peroxidase enzym in de cellen aan te tonen met behulp van een methode die de enzymatische activiteit bepaalt.

De belangrijkste conclusie uit dit onderzoek is dat de opname en intracellulaire route van Tf-gelabelde liposomen en Tf-enzym conjugaten verschillend is. De opname van de Tf-enzym conjugaten is niet alleen hoger dan de binding van de conjugaten aan de cellen, maar is ook hoger dan de opname van de Tf-liposomen. Dit heeft met name te maken met de grootte van de deeltjes. Zoals in figuur 3 schematisch is weergegeven, hebben Tf en het peroxidase een diameter van ca. 3 - 4 nm, terwijl de liposomen een diameter van 100 nm hebben. Dit grootte verschil veroorzaakt zeer waarschijnlijk het verschil in opname en intracellulaire bestemming (routing).



Figuur 3: Schematisch vergelijking tussen een Tf-enzym conjugaat en een Tf-gelabeld liposoom. Tf is weergegeven als een zwarte ovaal, peroxidase als een open rechthoek. De structuur van een liposoom is in deze figuur ook duidelijk te zien; het polyethylene glycol (PEG) op het oppervlak bevat een maleimide groep waaraan Tf gekoppeld kan worden.

Afhankelijk van het intracellulaire doel van het geneesmiddel is een Tf-geneesmiddel conjugaat dan wel een Tf-gelabeld liposoom meer geschikt voor geneesmiddeltoediening. Een belangrijk voordeel van liposomen is de mogelijkheid om meerdere geneesmiddelmoleculen per liposoom te vervoeren. Bovendien is het voor insluiting in liposomen niet nodig om deze geneesmiddelen te modificeren, zoals voor directe koppeling aan Tf vaak wel het geval is. Tf-gelabelde liposomen lijken bij uitstek geschikt voor de behandeling van lysosomale stapelingsziekten (bijvoorbeeld de ziekte van Gaucher of de ziekte van Pompe), waarbij het belangrijk is dat een geneesmiddel in de lysosomen terecht komt. Daarbij is het ook mogelijk om Tf-gelabelde liposomen te optimaliseren door bestanddelen te gebruiken die na een verlaging van de zuurgraad (zoals gebeurt na opname van Tf via clathrine-gecoate blaasjes) uit elkaar vallen, waardoor de inhoud van de liposomen vrijkomt. Vervolgens kunnen peptides aan de liposomen worden toegevoegd die zorgen voor ontsnapping uit de endosomen. Een bekend voorbeeld is het TAT peptide uit het HIV virus, dat zorgt voor ontsnapping van het virus uit de endosomen.

4.1. Liposomaal geneesmiddeltransport tijdens ontstekingen

Ontstekingen, zoals bijvoorbeeld bacteriële meningitis (hersenvliesontsteking) kunnen de functie van de BHB aantasten. Hierbij speelt lipopolysaccharide (LPS), wat op het oppervlak van gram-negatieve bacteriën aanwezig is, een belangrijke rol. Omdat het ook in deze situatie belangrijk is om geneesmiddelen naar de BHB en naar de hersenen te sturen, werd eerst de expressie van de TfR in aanwezigheid van LPS bepaald. Vervolgens werd het liposomale geneesmiddeltransport in aanwezigheid van LPS onderzocht.

De TfR dichtheid was in aanwezigheid van LPS niet veranderd, en ook de opname bleek ongewijzigd (**hoofdstuk 3**). In eerste instantie leek het aantal TfR als gevolg van LPS wel verlaagd te zijn, maar de resultaten waren beïnvloed door de toename in totale hoeveelheid cel eiwit. Om resultaten tussen experimenten te kunnen vergelijken is het gebruikelijk om de receptor-dichtheid per milligram of microgram eiwit uit te drukken. LPS veroorzaakt echter, na ca. 2 uur incuberen, een verhoging in de hoeveelheid cel eiwit door de aanmaak van zogenaamde acute fase eiwitten. Na 16 uur incubatie met

LPS was de eiwitconcentratie in de cellen weer genormaliseerd. Omdat bekend is dat LPS dit eiwitverhogend effect heeft, is ervoor gekozen om in deze gevallen de receptordichtheid te corrigeren voor de hoeveelheid cel eiwit van de controle situatie (geen LPS, uitgevoerd in hetzelfde experiment). Dit gaf aan dat de TfR ook in de aanwezigheid van een bacteriële ontsteking geschikt is voor geneesmiddelafgifte aan de hersenen.

Om het liposomale geneesmiddeltransport tijdens een ontsteking te onderzoeken werd gebruik gemaakt van endotheelcellen en astrocyten die ieder aan een kant van een poreus membraan werden gekweekt. Doordat de endotheelcellen in dit model zeer dicht tegen elkaar aan liggen, zoals bij de BHB het geval is, kan er een Trans-Endothele Elektrische Weerstand (TEER) gemeten worden. Na toevoeging van LPS wordt deze TEER verlaagd, doordat de openingen tussen de endotheelcellen groter worden. Het doel van dit onderzoek was dan ook om te testen of liposomen die een ontstekingsremmer, te weten N-acetyl-L-cysteïne, bevatten een verlaging van TEER tegen kunnen gaan. Deze ontstekingsremmer vangt schadelijke radicalen weg, maar heeft alleen in hele hoge concentraties effect, omdat het zeer moeilijk de cel binnenkomt. De verwachting was dat door het gebruik van Tf-gelabelde liposomen, een lagere concentratie ontstekingsremmer gebruikt kan worden, omdat Tf-gelabelde liposomen makkelijker de endotheelcellen binnen kunnen komen.

Na 16 uur incuberen met liposomen bleek inderdaad dat er geen verlaging van de TEER werd gemeten na toevoeging van LPS (**hoofdstuk 7**). Echter, liposomen die geen ontstekingsremmer bevatten hadden hetzelfde effect. Om dit onverwachte effect verder uit te zoeken werden in eerste instantie liposomen met LPS gemengd en na 2, 6 of 16 uur aan de endotheelcellen toegevoegd. Hieruit bleek dat het LPS dat minimaal 6 uur geïncubeerd was met liposomen geen verlagend effect had op de TEER. Vervolgens is met behulp van fluorescerend LPS vastgesteld dat na 6 uur incuberen met liposomen, ca. 15 – 20 % van het LPS was weggevangen door de liposomen.

Uit deze resultaten blijkt dat liposomen op zich geschikt zijn voor geneesmiddeltoediening tijdens ontstekingen, maar dat de liposomen ook een eigen effect hebben tegen LPS. Wel moet nog worden onderzocht of de interactie van liposomen met LPS de opname van liposomen door endotheelcellen beïnvloedt. Ook moet worden gekeken naar het gebruik van andere ontstekingsremmers, die krachtiger zijn dan N-acetyl-L-cysteïne. Tevens bestaat dan wellicht ook de mogelijkheid om Tf-

geneesmiddel conjugaten te gebruiken, wat in het geval van N-acetyl-L-cysteïne niet mogelijk was.

5. Toekomst perspectieven voor geneesmiddeltransport naar de hersenen

Het onderzoek beschreven in dit proefschrift heeft zich toegespitst op het gebruik van de TfR voor geneesmiddeltransport naar de hersenen. Hierbij is gebruik gemaakt van een *in vitro* BHB model, waardoor een meer mechanistische aanpak mogelijk was. Voor doelgerichte geneesmiddeltoediening is verder gebruik gemaakt van het natuurlijke ligand voor de TfR. Voor gebruik *in vivo* is dit wellicht niet mogelijk, omdat het al aanwezige Tf in bloed dan al zorgt voor een volledige receptor bezetting. Daarom is er veelvuldig onderzoek gedaan naar het gebruik van antilichamen tegen de TfR voor geneesmiddelafgifte. Deze binden aan de TfR op een andere plaats dan Tf, maar veroorzaken wel opname van de TfR door de endotheelcellen van de BHB. Het nadeel van deze antilichamen is de immunologische reactie die ze opwekken. Idealiter zou een vector gericht tegen de TfR een klein, niet immunologisch ligand moeten zijn, wat opname van de TfR door endotheelcellen initieert. Recent is er onderzoek gedaan naar fragmenten van antilichamen en kleine peptiden die binden aan de TfR, maar er kan ook worden gedacht aan een synthetisch ligand.

Behalve de TfR kan er ook gedacht worden aan andere receptoren op de BHB, zoals bijvoorbeeld de insuline receptor. Ook niet-receptor gemedieerde transport-mechanismen kunnen wellicht gebruikt worden voor geneesmiddeltransport naar de hersenen. Met een nieuwe technologie op het gebied van gen-expressie is het ook mogelijk om nieuwe "targets" te vinden (receptoren of andere transporters), die zeer specifiek tot expressie worden gebracht op de endotheelcellen van de BHB. Of wellicht targets die alleen onder bepaalde condities (bijv. een ziekte) tot expressie worden gebracht op de BHB.

Als alternatieve mogelijkheid voor geneesmiddeltransport naar de hersenen kan ook gedacht worden aan ziekteverwekkers. Bepaalde virussen, bacteriën, schimmels en parasieten zijn namelijk in staat om de BHB te passeren. Bacteriën bijvoorbeeld gebruiken cellen uit het bloed als "het paard van Troje" om de BHB te passeren. Een

ander voorbeeld zijn prionen (ziekte van Creutzfeld Jakob), die de BHB omzeilen door van andere transportroutes naar de hersenen gebruik te maken. Door deze processen te bestuderen kan men in de toekomst wellicht niet alleen nieuwe geneesmiddelen ontwikkelen tegen deze ziekteverwekkers, maar ook gebruik maken van deze transportmogelijkheden.

6. Conclusie

Het onderzoek beschreven in dit proefschrift draagt bij aan de ontwikkeling van nieuwe methoden voor selectief geneesmiddeltransport naar de hersenen. Hiervoor zijn, via een mechanistische aanpak, de mogelijkheden van de TfR onderzocht, waarbij gebruik is gemaakt van radioactief gelabeld Tf, Tf-enzym conjugaten en Tf-gelabelde liposomen. De efficiëntie van het geneesmiddeltransport hangt vooral af van de grootte van het deeltje: een Tf-enzym conjugaat wordt relatief makkelijk opgenomen door de TfR, terwijl de opname en de intracellulaire "routing" van Tf-gelabelde liposomen anders verloopt. De belangrijkste conclusie uit dit onderzoek is dat sturing van geneesmiddelen naar de hersenen via de TfR op de BHB mogelijk is.

List of Abbreviations

ACM	astrocyte conditioned medium
BBB	blood-brain barrier
BCEC	brain capillary endothelial cell
Da	Dalton
DFO	deferoxamine mesylate
DOPE	dioleoylphosphatidylethanolamine
EDTA	ethylenediaminetetraacetic acid
EPC-35	egg-phosphatidylcholine, partially saturated
HBS	HEPES buffered saline
HRP	horseradish peroxidase
IRE	iron responsive element
Lipo C	non-tagged liposomes
Lipo Tf	transferrin-tagged liposomes
LPS	lipopolysaccharide
mRNA	messenger RNA
NAC	N-acetyl-L-cysteine
NEM	N-ethylmaleimide
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEG-DSPE	1,2-diasterearoyl-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)2000]
PhAsO	phenylarsine oxide
p.i.	polydispersity index
PL	phospholipid
PMSF	phenylmethylsulfonylfluoride
RMT	receptor mediated transcytosis
SAINT	Synthetic Amphiphile INTeraction
SATA	N-succinimidyl-S-acetylthioacetate
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TEER	TransEndothelial Electrical Resistance
Tf	holo-transferrin (diferric transferrin)
TfR	transferrin receptor
TMB	3,3',5'5-tetramethylbenzidine
TL	total lipid
T-8	tyrphostin-A8

Curriculum Vitae

Corine Visser werd geboren op 3 september 1977 te Gorinchem, waar zij in 1995 aan de Christelijke scholengemeenschap "Oude Hoven" haar VWO diploma behaalde. Vervolgens begon zij in 1995 met de studie Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden, waar zij in 1997 het propaedeutisch diploma verkreeg. Tijdens de doctoraal-fase heeft zij, onder begeleiding van Dr. A.P. Kourounakis en Prof. Dr. A.P. IJzerman, onderzoek verricht naar de allostere modulatie van de Adenosine A_1 receptor. Vervolgens heeft zij bij SmithKline & Beecham (nu GlaxoSmithKline), Harlow, Engeland, onderzoek gedaan op de afdelingen "high-throughput screening" en "new assay technologies", onder begeleiding van Drs. K. Dodgson en Dr. M Rüdiger. In Maart 2000 werd het doctoraal diploma Bio-Farmaceutische Wetenschappen behaald.

Van maart 2000 tot en met mei 2004 was zij assistent in opleiding bij de Blood-Brain Barrier Research group van de divisie Farmacologie van het Leiden/Amsterdam Center for Drug Research (LACDR) onder begeleing van Dr. A.G. de Boer, Prof. Dr. M. Danhof en Prof. Dr. D.J.A. Crommelin (Afdeling Biofarmacie, Utrecht Institute for Pharmaceutical Sciences (UIPS), Universiteit Utrecht). Het onderzoek beschreven in dit proefschrift is uitgevoerd als onderdeel van het NDRF-project "Targeting of CRH-receptor antisense probes to and in the central nervous system. Application in neuropsychiatry", een samenwerkingsverband tussen de Universiteit Groningen, Solvay Pharmaceuticals en de afdeling Farmacologie van het LACDR.

Vanaf 1 september 2004 is de auteur van dit proefschrift als Scientist CNS targeting werkzaam bij to-BBB technologies B.V. te Leiden.

List of Publications

PJ Gaillard, CC Visser, AG de Boer, Targeted delivery across the blood-brain barrier, an invited review for Expert Opinion on Drug Delivery, *in preparation*

CC Visser, LH Voorwinden, L van Bloois, DJA Crommelin, M Danhof and AG de Boer, Interaction of liposomes with lipopolysaccharide: influence of time of incubation, presence of serum and liposomal composition, *submitted for publication*

CC Visser, S Stevanović, LH Voorwinden, PJ Gaillard, M Danhof, DJA Crommelin and AG de Boer, Liposomal targeting of proteins to the blood-brain barrier *in vitro, submitted for publication*

CC Visser, LH Voorwinden, L van Bloois, L Harders, M Eloualid, DJA Crommelin, M Danhof and AG de Boer, Coupling of metal containing homing devices to liposomes via a maleimide linker: use of TCEP to stabilize thiol-groups without scavenging metals, *Journal of Drug Targeting, in press*

AG de Boer, CC Visser and PJ Gaillard, Elke barrière heeft zijn bres*, Farmaceutisch Weekblad*, (2004) 139: 948-953 (in English: Each barrier has its breach, *Dutch Weekly Pharmaceutical Journal*)

CC Visser, S Stevanović, LH Voorwinden, PJ Gaillard, DJA Crommelin, M Danhof and AG de Boer, Validation of the transferrin receptor for drug targeting to brain capillary endothelial cells *in vitro*. *Journal of Drug Targeting* (2004) 12 (3): 145 - 150

CC Visser, LH Voorwinden, DJA Crommelin, M Danhof and AG de Boer, Characterisation and modulation of the transferrin receptor on brain capillary endothelial cells, *Pharmaceutical Research* (2004) 21 (5): 761 - 769

AP Kourounakis, C Visser, M de Groote and AP IJzerman, Differential effects of the allosteric enhancer PD81,723 on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A_1 receptor, *Biochemical Pharmacology* (2001) 61: 137-144

AP Kourounakis, C Visser, M de Groote and AP IJzerman, Allosteric modulation of the rat adenosine A_1 receptor: differential effects on agonist and antagonist binding, *Drug Development Research* (2000) 51: 207-215

Nawoord

Het nawoord van een proefschrift is wellicht het gedeelte waar de meeste clichés al over gezegd zijn. Toch ben ook ik iedereen die op zijn of haar manier een bijdrage geleverd heeft aan de totstandkoming van mijn proefschrift zeer erkentelijk!

Een aantal mensen wil ik echter in het bijzonder noemen. Allereerst Heleen Voorwinden, die door haar inzet een zeer belangrijke bijdrage geleverd heeft aan mijn promotieonderzoek. Fijn dat je in de slotfase mijn paranimf wilt zijn!

Pieter Gaillard ben ik zeer erkentelijk voor zijn adviezen en Arjen Brink voor zijn hulp met de experimenten. De inzet van de stagestudenten Mohamed Eloualid, Liesbeth Harders en Sanja Stevanvović is voor een groot deel terug te vinden in dit proefschrift. Voor mijn experimenten heb ik gebruik gemaakt van apparatuur en kennis van zo ongeveer alle LACDR afdelingen. Ik heb de hulp van alle LACDR medewerkers dan ook zeer gewaardeerd. De AAS metingen die Jos van Brussel (LIC) uitgevoerd heeft wil ik hierbij niet onvernoemd laten.

Met alle (oud-)collega's van de afdeling Farmacologie heb ik met veel plezier samengewerkt, vooral met de AIO's. De hulp van Sandra Visser gedurende mijn eerste aio-maanden heb ik zeer gewaardeerd en ook Dymphy Huntjens wil ik vermelden voor haar hulp met NONMEM. Met Lia Liefaard heb ik al vanaf onze BFW-studie veel plezierige uren op en buiten het lab doorgebracht. Leuk dat jij nu ook mijn paranimf wilt zijn!

Op de afdeling Biofarmacie van de Universiteit Utrecht heb ik altijd met veel plezier liposomen gemaakt. Vooral de hulp van Louis van Bloois, Gerben Koning en Marjan Fretz heb ik zeer gewaardeerd. De discussies met alle deelnemers van Solvay Pharmaceuticals en de Universiteit van Groningen tijdens het twee-jaarlijks NDRF-overleg waren vaak pittig. Ik heb daar veel van geleerd!

Mijn vrienden en familie ben ik zeer dankbaar voor hun steun en interesse. Angeliki, thank you for your introduction to science and for the friendship we share since then. Oma Hol, Oma Visser, Bart, Attie & Frank, Willeke & Marcel, Mariet & Eisse-Piet, Heidi & John wil ik in het bijzonder noemen voor hun steun en interesse, alsook voor vele gezellige momenten!

Mijn ouders wil ik bedanken, die mij gestimuleerd en gesteund hebben bij alles wat ik doe. Ik draag dit proefschrift dan ook met veel plezier aan jullie op! Tot slot Vincent, jouw liefde, steun en optimisme heeft me door de laatste fasen van het onderzoek heen gesleept. "Alles sal reg kom!", waar we samen ook heen gaan.

Corine