



Universiteit
Leiden
The Netherlands

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

Visser, Corine

Citation

Visser, C. (2005, January 18). *The Transferrin Receptor at the Blood-Brain Barrier - exploring the possibilities for brain drug delivery*. Retrieved from <https://hdl.handle.net/1887/586>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/586>

Note: To cite this publication please use the final published version (if applicable).

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% CO₂ 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% CO₂ 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 FeCl₃ 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 H₂SO₄ 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, $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 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 $\mu\text{g}/\text{ml}$ Tf-HRP at 37 °C showed a concentration-dependent, specific association (a combination of binding and endocytosis), which was linear up to 10 $\mu\text{g}/\text{ml}$ (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 $0.051 \pm 0.014 \text{ min}^{-1}$. 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 $\mu\text{g}/\text{ml}$ 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.

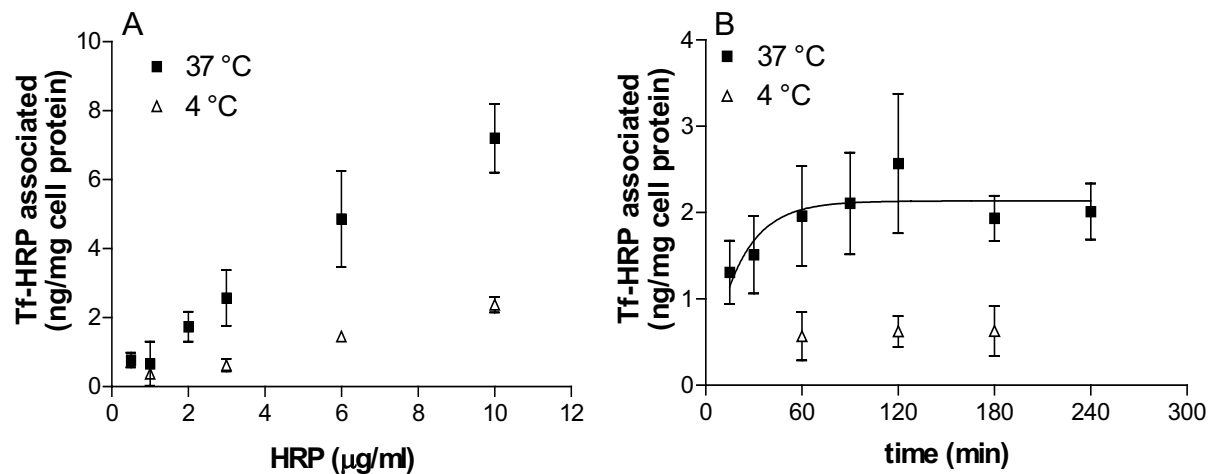


Figure 1: Concentration (A) and time (B) dependent association and binding of Tf-HRP. Incubation of BCEC with Tf-HRP (0.5 – 10 $\mu\text{g/ml}$) for 2 h at 37 °C or at 4 °C showed a concentration dependent association of Tf-HRP, which is linear up to 10 $\mu\text{g/ml}$. The association at 37 °C was approximately 4-fold higher at each concentration tested. Incubation of BCEC with 3 $\mu\text{g/ml}$ 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 $\pm$ 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 $\mu\text{g/ml}$ 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).

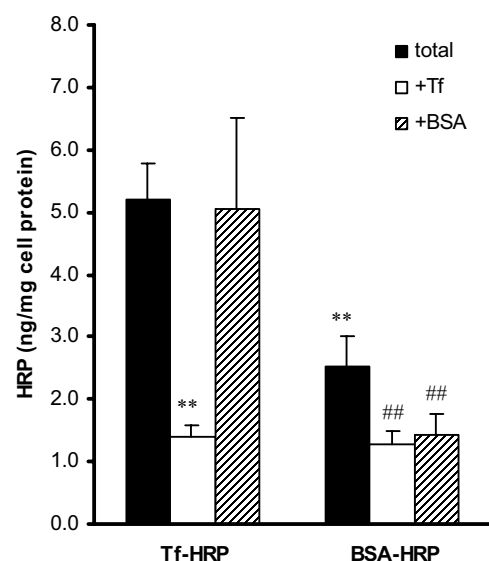


Figure 2: Association of Tf-HRP (3 $\mu\text{g}/\text{ml}$, 2 h at 37 $^{\circ}\text{C}$) was reduced by a 500-fold excess of non-conjugated Tf, but not by an equal concentration of BSA. Total association of BSA-HRP (3 $\mu\text{g}/\text{ml}$, 2 h at 37 $^{\circ}\text{C}$) was approximately 50 % of the association of Tf-HRP, and was inhibited by both Tf and BSA. Data represented are mean $\pm$ 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 $^{\circ}\text{C}$, 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).

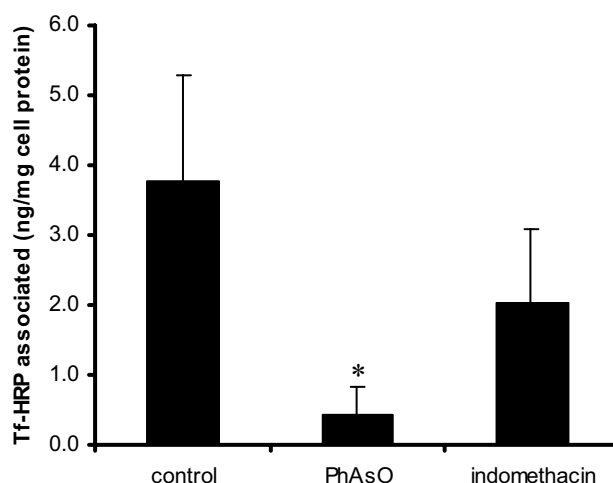


Figure 3: BCEC were incubated with 3 $\mu\text{g}/\text{ml}$ Tf-HRP for 1 h. The specific association of Tf-HRP was inhibited by PhAsO (1 μM), but not by indomethacin (50 $\mu\text{g}/\text{ml}$), indicating that endocytosis of Tf-HRP occurs via clathrin-coated vesicles. Data represented are mean $\pm$ 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.

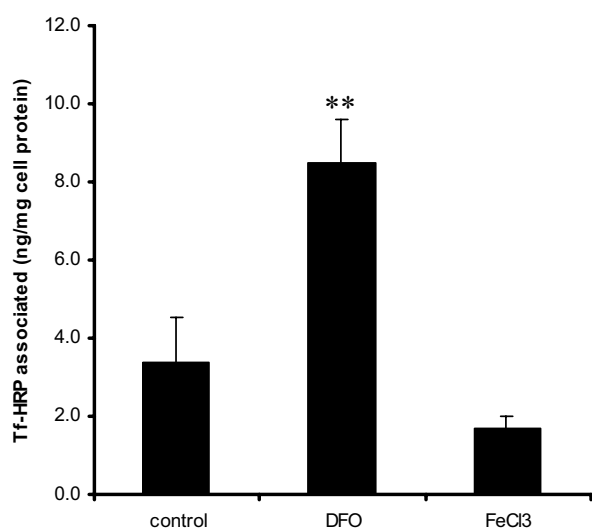


Figure 4: BCEC were incubated with 3 $\mu\text{g}/\text{ml}$ Tf-HRP for 2 h at 37 °C, after 24 h pre-incubation with DFO or FeCl_3 (both 1 mM). DFO caused a 2.5-fold increase in the specific association of Tf-HRP while FeCl_3 had no significant effect. Data represented are mean $\pm$ SD ($n = 3$ experiments, 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 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$) and Tf (3 $\mu\text{g}/\text{ml}$ 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

