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

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Chapter 3

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

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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).

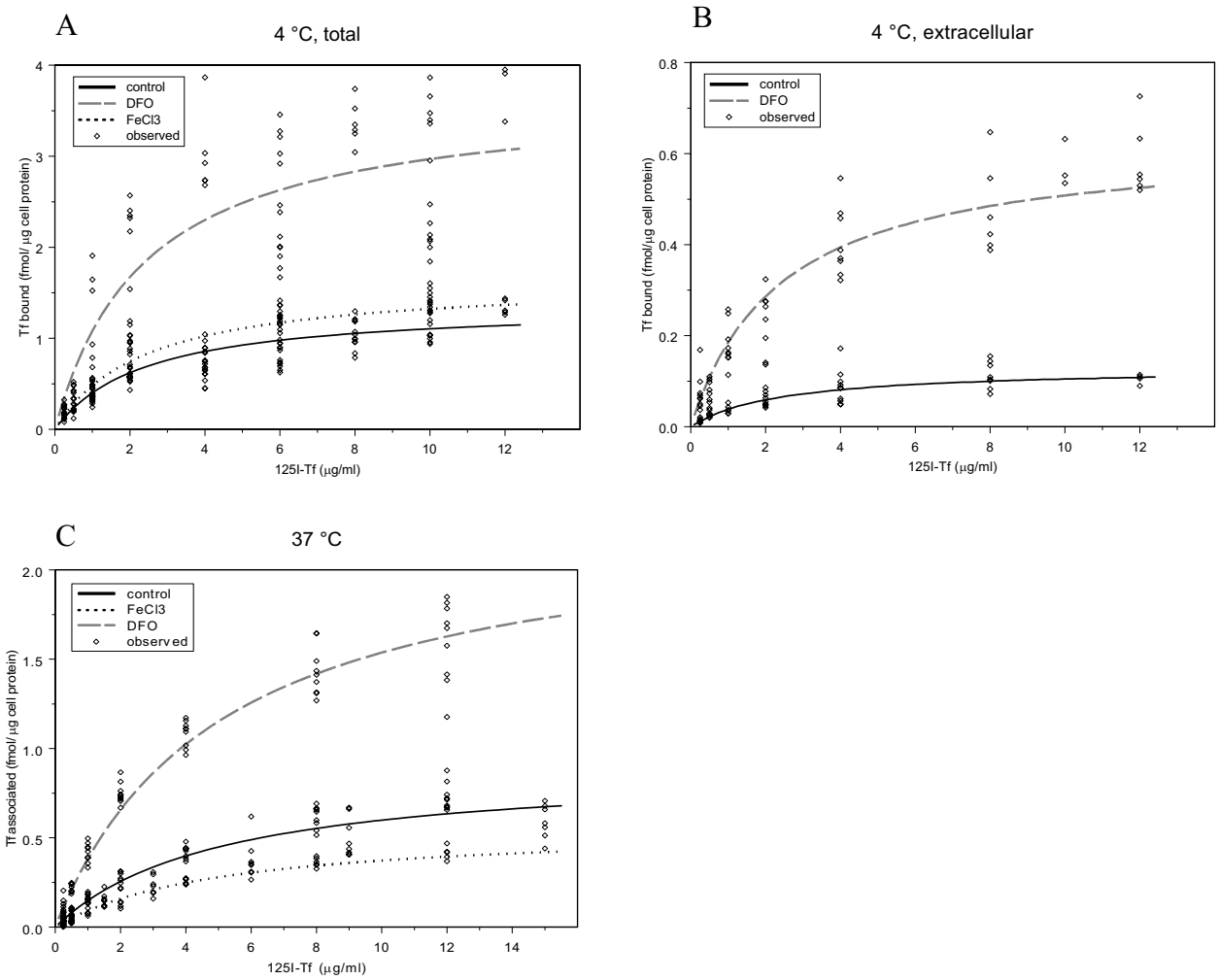

Figure 1: Total (**A**) and extracellular binding (**B**) of ^{125}I-Tf to the TfR, determined at 4 °C, and association (**C**) of ^{125}I-Tf by the TfR, determined at 37 °C, on primary cultured BCEC in the presence of DFO or $FeCl_3$ (1 mM). Data represented are the curves of specific binding, from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

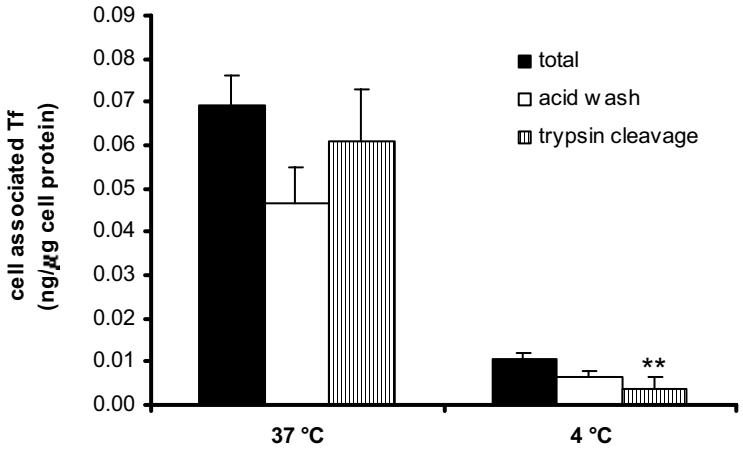

Figure 2: Cell-associated ^{125}I-Tf at 37 °C was reduced 20 – 30 % after acid wash (citric acid, pH 5) or trypsin cleavage (0.25 mg/ml trypsin), indicating that 70 – 80 % of the ^{125}I-Tf was endocytosed. At 4 °C the binding of ^{125}I-Tf is reduced to the level of non-specific binding after trypsin 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.

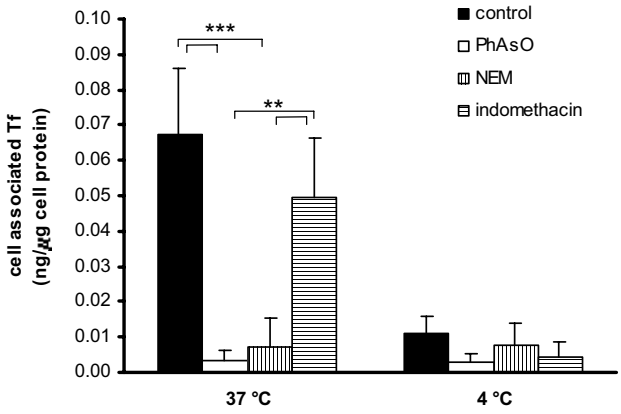

Figure 3: Endocytosis, determined at 37 °C, was inhibited after incubation with PhAsO (10 µM) and NEM (1 mM), but not after incubation with indomethacin (50 µg/ml), indicating that clathrin-mediated endocytosis is involved. At 4 °C non of these inhibitors had an effect on the extracellular binding of ^{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).

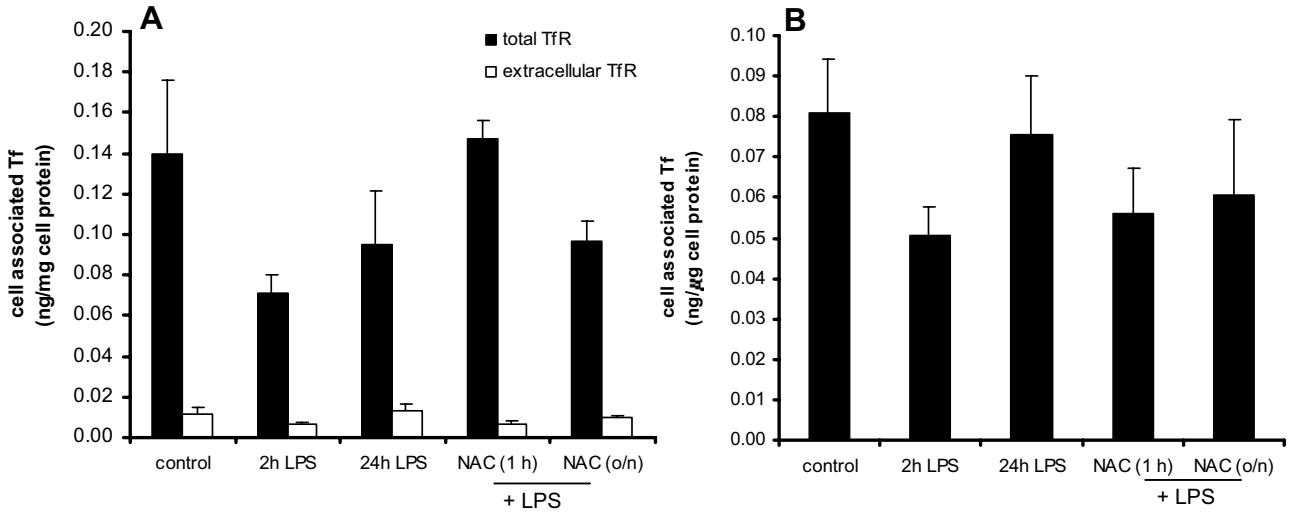

Figure 4: Total and extracellular binding (**A**) of ^{125}I-Tf to the TfR, determined at 4 °C, and association (**B**) of ^{125}I-Tf by the TfR, determined at 37 °C, after pre-incubation with 100 ng/ml LPS for 2 or 24 h, or after combined pre-incubation with 10 mM NAC (1 h or overnight), followed by 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).

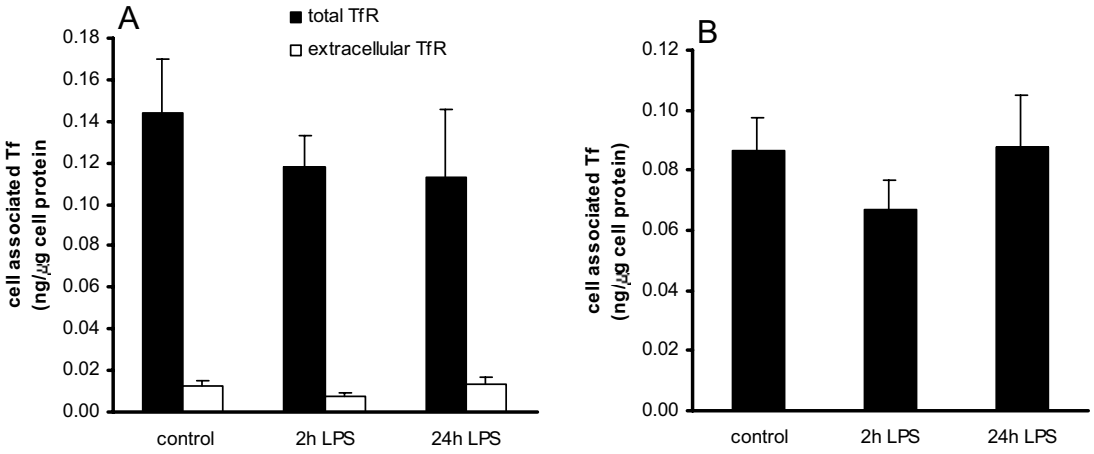

Figure 5: Total and extracellular binding (**A**) of ^{125}I-Tf to the TfR, determined at 4 °C, and endocytosis (**B**) of ^{125}I-Tf by the TfR, determined at 37 °C, after pre-incubation with 100 ng/ml LPS (2 or 24 h) and expressed in ng cell-associated Tf corrected for cellular protein levels of the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

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