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Receptor-mediated endocytosis of transferrin at the blood-brain barrier

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SUMMARY

Rat brains were perfused with a transferrin-peroxidase conjugate (Tf-HRP) to characterize morphologically the endocytic pathway of transferrin in blood-brain barrier endothelial cells. Electron microscopic evaluation of rat brains perfused with Tf-HRP at 4˚C and subsequently warmed to 37˚C for brief periods of time (2 minutes) showed sequestration of Tf-HRP in clathrin coated pits and vesicles on the luminal membrane of the brain endothelium. After 5 minutes of warming, diaminobenzidine (DAB) reaction product was present in vesicular structures 250-500 nm in diameter and in associated tubules morphologically identified as large or sorting endosomes. Recycling endosomes were also heavily labelled at this time point. Almost no DAB reaction product remained in the cerebral endothelial cells when the warming period was longer than 15 minutes. Other rat brains were perfused with Tf-HRP at 30˚C for 15 minutes prior to fixation and DAB cytochemistry. In these studies, brain endothelial cells contained large amounts of DAB reaction product, mostly localized in 50-100 nm vesicles and tubules, often in the Golgi region of the cells. Coated pits and vesicles and large endosomes were also heavily labelled. Transcytosis of Tf-HRP was not identified in either perfusion protocol. Ultrastructural, indirect immunocytochemical localization of transferrin receptors showed that the transferrin receptor is highly polarized at the blood-brain barrier and is localized only on the apical membrane, in contrast to other polarized epithelial cells, like hepatocytes, in which the receptor is present on the basolateral membrane. The evidence supports an iron transport model in which iron-loaded transferrin is taken up by receptor-mediated endocytosis at the luminal membrane of brain capillaries. The iron then dissociates from transferrin in endosomal compartments and is transcytosed by unknown mechanisms, while the transferrin is retroendocytosed.

Key words: transferrin, blood brain barrier, endocytosis, endothelium

INTRODUCTION

The endothelial cells of the brain comprise the blood-brain barrier (BBB) (Bradbury, 1985; Broadwell, 1989). These cells function as a tight epithelium and prevent the uncontrolled transport of water-soluble molecules between vascular elements and the brain parenchyma (Fenstermacher and Rappoport, 1984; Risau et al., 1990). The endothelial cells at the BBB possess at least two structural features that are critical for this barrier function. Complex, tight intercellular junctions are located between adjacent cells and effectively obliterate the lateral intercellular space (Shrivers et al., 1984), thus preventing paracellular solute transport between adjacent cells. Also, pinocytotic vesicular activity in BBB endothelial cells is extremely rare, as is fluid phase transcellular transport (Reese and Karnovsky, 1967). These characteristics greatly restrict the uncontrolled permeation of hydrophilic molecules, including glucose and amino acids across the BBB. Capillary endothelial cells in the brain exhibit various specific transport proteins, including glucose and amino transporters, that mediate biologically essential molecules across the BBB into the brain (Pardridge and Oldendorf, 1975a,b, 1977).

Cells in the central nervous system require iron and it is not clear how this element crosses the BBB. Iron circulates in the blood bound to transferrin, an 80 kDa glycoprotein, which distributes iron to the tissues (Aisen and Listowsky, 1980; Hanover and Dickson, 1985). Transferrin binds ferric ions with very high affinity at neutral pH and low affinity in acidic conditions (Baldwin et al., 1982). Transferrin receptors are present on the surface of a diverse assortment of cell types and mediate the internalization of iron-saturated transferrin by receptor-mediated endocytosis (Octave et al., 1983; van der Ende et al., 1987; Willingham et al., 1984). Studies have shown that the affinity of transferrin for its receptor depends on pH and iron loading (Dautry-Varsat, 1983; Klausner et al, 1983). These properties are important because internalized transferrin is routed through acidic intracellular compartments allowing selective accumulation of iron (Octave et al., 1983; van Renswoude et al., 1982).

Transferrin receptors are present on the luminal mem-

brane of brain endothelial cells and mediate the endocytosis of transferrin in these cells (Fishman et al., 1987; Jeffries et al., 1984; Pardridge et al., 1987; Friden et al., 1991). It is not known whether iron crosses the BBB bound to transferrin, or whether it dissociates from its carrier in endothelial cell endosomes and is subsequently transported into the brain by another mechanism. Taylor and Morgan(1990) have reported that most iron is released from transferrin in brain endothelial cells and nearly all endocytosed transferrin is rapidly recycled to the blood. Several other studies suggest that these receptors mediate the transcytosis of transferrin from blood to brain (Fishman et al., 1987; Jeffries et al., 1984; Pardridge et al., 1987; Friden et al., 1991). Brain endothelial cells are functionally a polarized epithelium with distinct apical and basal membrane domains (Beck et al., 1986; Betz et al., 1980). The endocytotic pathway of transferrin in polarized epithelial cells has been the focus of several reports and in these cells, transferrin receptors are usually expressed only on the basolateral membrane (Fuller and Simons, 1986; Hughson and Hopkins, 1990; Levine and Woods, 1990). The polarized distribution of this membrane protein is maintained, in part, because after endocytosis and endosomal processing the transferrin-receptor complex is directed back to the basal membrane (Fuller and Simons, 1986; Hughson and Hopkins, 1990). Transcytosis of transferrin has not been described in endothelium. Moreover, if transferrin transcytosis were to occur in brain endothelium, it is not clear that iron would accompany transferrin across the endothelial barrier. Efficient iron transport would require that the intracellular pathway does not include acidic endosomal compartments; however, a receptor-mediated endocytic pathway that bypasses endosomal processing is unprecedented.

The purpose of these studies was to characterize at the fine structural level the endocytic pathway of transferrin in cerebral endothelial cells. In the conditions used in this study, the transferrin endocytic pathway was found to be similar to that described for other polarized cells and direct transcytosis of transferrin across the BBB was not observed. Studies were also performed using MRC OX-26, an anti-transferrin receptor monoclonal antibody (Jeffries et al., 1984),to characterize the distribution and biochemical characteristics of the BBB transferrin receptor.

MATERIALS AND METHODS

Preparation of transferrin-peroxidase (Tf-HRP)

Rat transferrin (Tf) (Jackson Labs) was iron loaded and iodinated using the methods described by Yamashiro et al. (1984). Tf was covalently conjugated to peroxidase utilizing the hetero-bifunctional cross-linking agent *N*-succinimidyl 3-(2-pyridyldithio) proprionate (SPDP) (Carlsson et al., 1978), using the procedure recommended by the manufacturer (Pierce Chemical Co.). Briefly, 10 mg rat Tf in 1 ml PBS (pH 7.5) containing a trace amount of ^{125}I-Tf was reacted with 20 μg SPDP at room temperature for 30 minutes. The reaction products were passed over a PD-10 column and the protein peak containing Tf-PDP was collected. In a separate tube, 20 μg of SPDP was added to 5 mg horseradish peroxidase (HRP) in 1 ml of PBS and allowed to react for 30 minutes at room temperature. PDP-HRP was isolated after passage of the products over a PD-10 column. To introduce free sulfhydral groups in Tf-PDP, the solution was reduced to pH 6.0 and dithiothreitol was added to a final concentration of 10 mM. After 30 minutes at room temperature the products, including Tf-SH, were passed over a PD-10 column and the protein peak recovered. The PDP-HRP was then added to the Tf-SH solution and the pH adjusted to 7.6. The solution volume was reduced to 0.5 ml using a Centricon PM10 concentrator and the reaction allowed to continue for 12 hours at room temperature. The reaction products were analyzed by non-reducing SDS-PAGE and autoradiography. Tf-HRP was separated from unreacted PDP-HRP and Tf-SH by passage over a Sephacryl 200 column.

Perfusion protocols

21-day-old Sprague-Dawley rats were euthanized with ether and the vascular system was cleared of blood elements by perfusion through the left ventricle with warm (30˚C), oxygenated M199 (with 20 mM Hepes, pH 7.4) containing 2 mg/ml bovine serum albumen. Two different perfusion protocols were followed in these studies. A temperature-shift protocol allowed synchronization of the endocytic process and analysis of the sequential compartments to which Tf-HRP was directed in the endothelium. In the other perfusion protocol, rat brains were perfused with Tf-HRP (1 mg/ml) for up to 15 minutes at 30˚C. This allowed analysis of the transferrin pathway under conditions approaching steady state and facilitated the uptake of greater amounts of Tf-HRP. In the temperature-shift experiments, after clearing of the blood elements as described above, the animals were cooled to 4˚C by placing them in an ice bath and perfusing with cold M199 for 5 minutes. The ascending aorta was then identified and canulated and all subsequent perfusion occurred through this vessel at a flow rate of 5 ml/minute. Following perfusion with cold buffer containing Tf-HRP (100-200 μg/ml) for 15 minutes to allow for receptor-ligand binding, the animals were placed in a 37˚C bath and further perfused for various lengths of time with oxygenated buffer warmed to 37˚C. The animals were finally perfused with fixative containing 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer at room temperature. Nonspecific (non-transferrin receptor-mediated) endocytosis of Tf-HRP was determined in control experiments in which native rat ferrotransferrin (5 mg/ml) was added to the perfusate to compete with Tf-HRP. Biochemical analysis of the transferrin endocytic pathway in cerebral endothelium was done by repeating the protocol described above but substituting ^{125}I-transferrin (10^5 cts per min/ml) for Tf-HRP in the perfusate. After various times at 37˚C, isolated brains were subjected to quantification of transferrin uptake by gamma counting. Controls for these experiments included 1 mg/ml native rat transferrin in the perfusate. Three animals were used for each time point and the results are expressed as the mean. Standard errors varied by less than 10% of the mean value.

Tissue processing for electron microscopy

After fixation brains were removed and post-fixed using the same fixative for 1 hour. Coronal sections, 50 μm thick, were cut with a vibratome and the peroxidase reaction was initiated by incubation of the sections in 50 mM phosphate buffer containing 0.01% H_2O_2 and 0.05% diaminobenzidine. After 10 minutes the sections were washed in buffer and processed for electron microscopy. Brain sections were flat embedded in Epon between opposed sheets of aclar plastic and then were remounted on polymerized blocks of epon using epoxy resin. These preparations were thin sectioned for electron microscopy. Sections were stained with lead citrate and photographed using a Hitachi H-7000 electron microscope operating at 50 kV.

In the temperature shift experiments, control perfusions (competition experiments) were done in five animals. In addition, the brains of five animals were examined ultrastructurally after each

of the following warming intervals after the perfusion of Tf-HRP in the cold: 2 minutes, 5 minutes, 10 minutes and 15 minutes. No warming interval longer than 15 minutes was included in the study because very few cerebral capillaries contained DAB reaction product after this period of warming. In addition two animals were examined after 4 minutes and 8 minutes respectively. In each animal perfused with Tf-HRP, 40 micrographs (20 micrographs of the 15 minutes of warming time point) of endothelial cells containing DAB reaction product were generated from each animal. Thus approximately 200 micrographs were generated for each time point included in the study. Micrographs were systematically taken from the same areas of 200 mesh grids and the grids were coded to minimize observer bias. The final magnifications of the electron micrographs ranged from 10,000-20,000×. In order to avoid regional variation in BBB transferrin receptor numbers, all micrographs were taken of thalamic capillaries. Other areas of the brain examined included the cerebral and cerebellar cortices, and although quantitative data were not generated from these regions of the brain, no qualitative differences were observed in the intracellular transferrin pathway.

Immunolocalization of transferrin receptors in rat brain

Transferrin receptors were localized by the procedure described by Levine and Woods (1990). Briefly, brains of anesthetized rats were fixed by vascular perfusion with periodate-lysine-paraformaldehyde (2% paraformaldehyde, 0.075 M lysine, 0.01 M $NaIO_4$, 0.0375 M $NaPO_4$, pH 6.2) (McLean and Nakane, 1974). The brains were removed and immersed for an additional 2 hours in the same fixative. Coronal sections 2-3 mm thick were cut, and sections of the thalamus further cut into 2-3 mm squares were washed in PBS containing 10% DMSO for 1 hour. The tissue was mounted on small sections of cork using O.C.T. compound and plunged into a propane slurry cooled by liquid nitrogen. 10 μm frozen sections were cut, picked up on coverslips, and subjected to indirect immunoperoxidase staining using MRC OX-26 anti-transferrin receptor monoclonal antibody (Jeffries et al., 1984) and a mouse Vectastin kit (Vector Labs, Burlingame, CA) using procedures recommended by the manufactures. Negative control experiments were performed using anti-fibronectin monoclonal antibodies in place of MRC OX-26. After these steps, the sections were fixed for 1 hour in 1% glutaraldehyde in 0.1 M phosphate buffer and then washed three times for 1 hour. The peroxidase reaction was initiated by incubating the sections in 50 mM phosphate buffer containing 0.01% H_2O_2 and 0.05% diaminobenzidine. After 10 minutes the sections were washed in buffer and either examined directly by light microscopy or processed for electron microscopy as described above. For immunogold labelling, after incubation in OX-26, cryosections were incubated for 1 hour at room temperature with gold-conjugated secondary antibody (Sigma Chemical Co., St. Louis, MO) diluted 1:25 in PBS. The sections were rinsed three times in PBS and processed for electron microscopy. For embedding, the sections on coverslips were mounted on the open end of Epon-filled beem capsules and polymerized overnight. The coverslips were separated from the tissue sections by immersion in liquid nitrogen. Thin sections were cut and photographed as described above.

RESULTS

Properties of Tf-HRP

Non-reducing SDS-polyacrylamide gels of the reaction products of the Tf-HRP preparation showed that SPDP efficiently mediates the covalent coupling of ^{125}I-transferrin and peroxidase. The major product showed a molecular weight of 105-110 kDa which represented monomeric Tf-HRP. Less prominent bands ran at higher molecular weights and most likely represented transferrin linked to more than one peroxidase molecule (data not shown). The biological activity of newly synthesized ^{125}I-Tf-HRP was assessed by competition experiments performed in the rat L6 cultured muscle cell line. In these studies the binding and internalization of ^{125}I-Tf-HRP was inhibited by excess native rat transferrin (Fig. 1). In other studies L6 cells were allowed to internalize Tf-HRP (100 μg/ml) for 30 minutes and then placed in fresh media without Tf-HRP for 60 minutes. The medium was collected and cells were fixed and processed for DAB cytochemistry and electron microscopy. Cells subjected to this experimental protocol contained very little DAB reaction product. In particular, no DAB reaction product was observed in lysosomes. Intact Tf-HRP was detected in the medium by subjecting it to non-reducing polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose and reaction with DAB (data not presented). As demonstrated in other systems (Stoorvogel et al., 1988), these experiments show that Tf-HRP is a biologically relevant probe as it specifically binds to transferrin receptors and is recycled back to the cell surface like native transferrin and not directed to lysosomes like transferrin-gold (Neutra et al., 1985).

Morphological analysis of Tf-HRP uptake in brain endothelium in situ

Electron microscopic examination of rat brains perfused with Tf-HRP (100-200 μg/ml) at 4°C for 15 minutes and then processed for DAB cytochemistry, revealed the presence of DAB reaction product on the luminal membrane of the cerebral endothelial cells in all areas of the brain examined including thalamus, cerebral cortex and cerebellar cortex. The reaction product was present within clathrin-coated regions of the luminal membrane domain. DAB reaction product was notably not associated with non-clathrin-coated pits or vesicles on the luminal membrane (Fig. 2a, inset). In negative control experiments, assessing

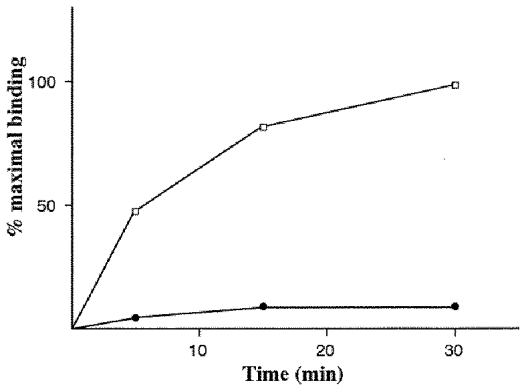

Fig. 1. Competition assay of ^{125}I-transferrin-HRP binding to L-6 cells in culture. ^{125}I-Tf-HRP (10 μg/ml) was incubated with cells for the indicated times in the presence (●) and absence (□) of 1 mg/ml Tf-HRP. Results represent the average of three separate determinations.

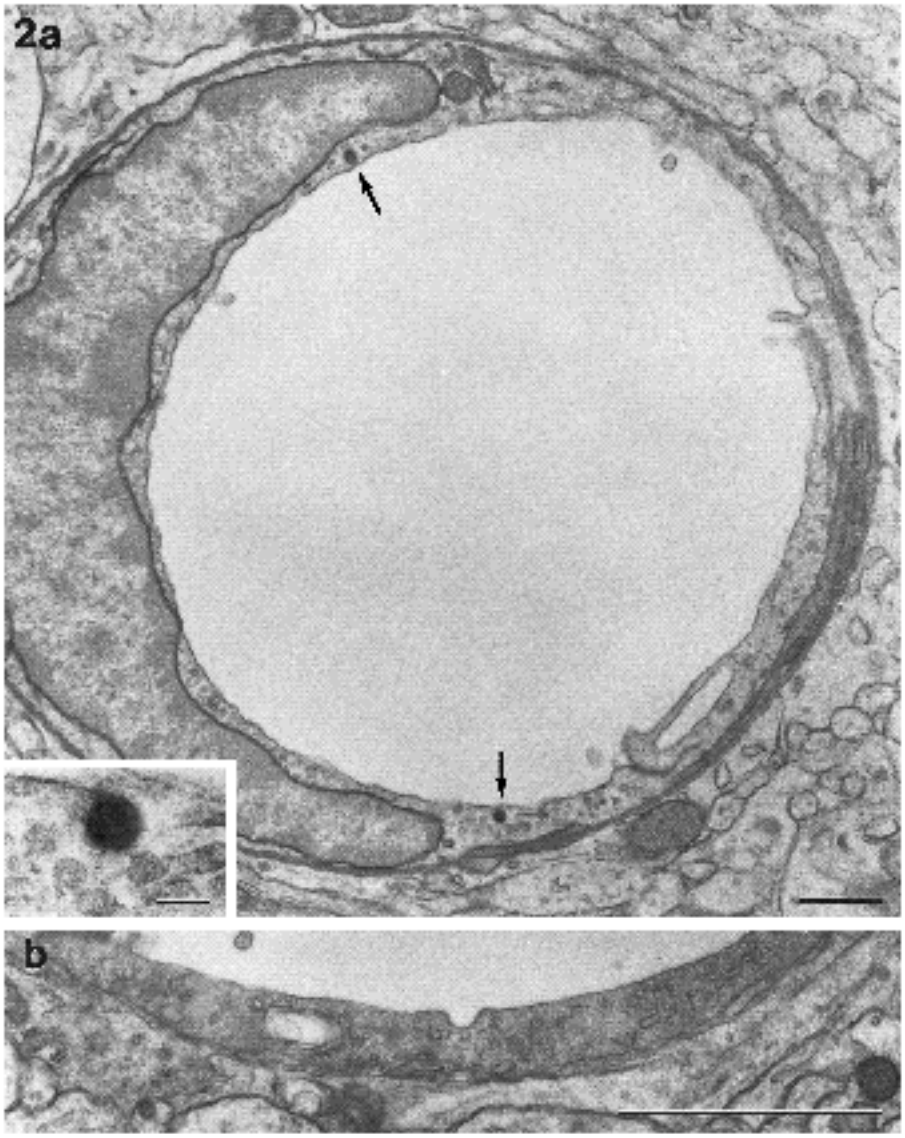

Fig. 2. Localization (arrows) of Tf-HRP in rat brain capillaries after perfusion at 4˚C for 15 min. (a) Survey electron micrograph showing a few labeled, coated vesicles. Bar, 1.0 μm. Inset: coated vesicle labeled with Tf-HRP at higher power. Bar, 0.1 μm. (b) control perfusion without Tf-HRP demonstrating no HRP reaction product within the endothelium. Bar, 1 μm.

the localization of endogenous peroxidase, endothelial cell-associated DAB reaction product was very rarely encountered and where identified was exclusively associated with undifferentiated aspects of the luminal membrane (Fig. 2b). No general, non-specific HRP labeling of endothelium was detected.

Other animals were perfused as described above, but were then subjected to a temperature-shift. This was done by perfusion of the animals with warmed Tf-HRP-free buffer for various time intervals prior to fixation, DAB cytochemistry and electron microscopy. After 2 minutes of warming, the DAB reaction product was primarily localized in coated pits on the endothelial cell surface and in cytoplasmic coated vesicles (Fig. 3a,b). With warming, very little reaction product remained over undifferentiated regions of the luminal membrane, indicating the Tf-HRP-receptor complexes are trapped in clathrin-coated membrane domains in preparation for internalization after short periods of warming. Non-clathrin-coated vesicles 200-400 nm in diameter also frequently contained DAB reaction product after 2 minutes of warming (Fig. 3c). These vesicles often consisted of an electron-lucent central region surrounded by DAB reaction product, and were structurally very similar to early endosomes as described in other reports (Gruenberg et al., 1989; Parton et al., 1989). Early endosomes have been shown by Yamashiro and Maxfield (1987) to be only mildly acidic with a luminal pH of 6.2-6.3. After 5 minutes of warming, most of the DAB reaction product was localized in 250-500 nm vesicles identified morphologically as sorting endosomes (Fig. 4a). This endosomal subtype is often referred to as the receptosome or CURL (Geuze et al., 1984; Willingham et al., 1984). Also labeled at this time point were 50-100 nm tubular (Fig. 4c) and vesicular structures (Fig. 4b,d), often in close proximity to profiles of the Golgi complex or the luminal membrane. The DAB-labelled tubulovesicles in endothelial cells were structurally similar to transferrin-containing structures, identified in other cell types (Willingham et al., 1984; Yamashiro et al., 1984) as recycling endosomes. At 10-15 minutes of warming the remaining Tf-HRP was mostly located in 50-100 nm, non-clathrin-coated vesicles that were often associated with the luminal membrane of the

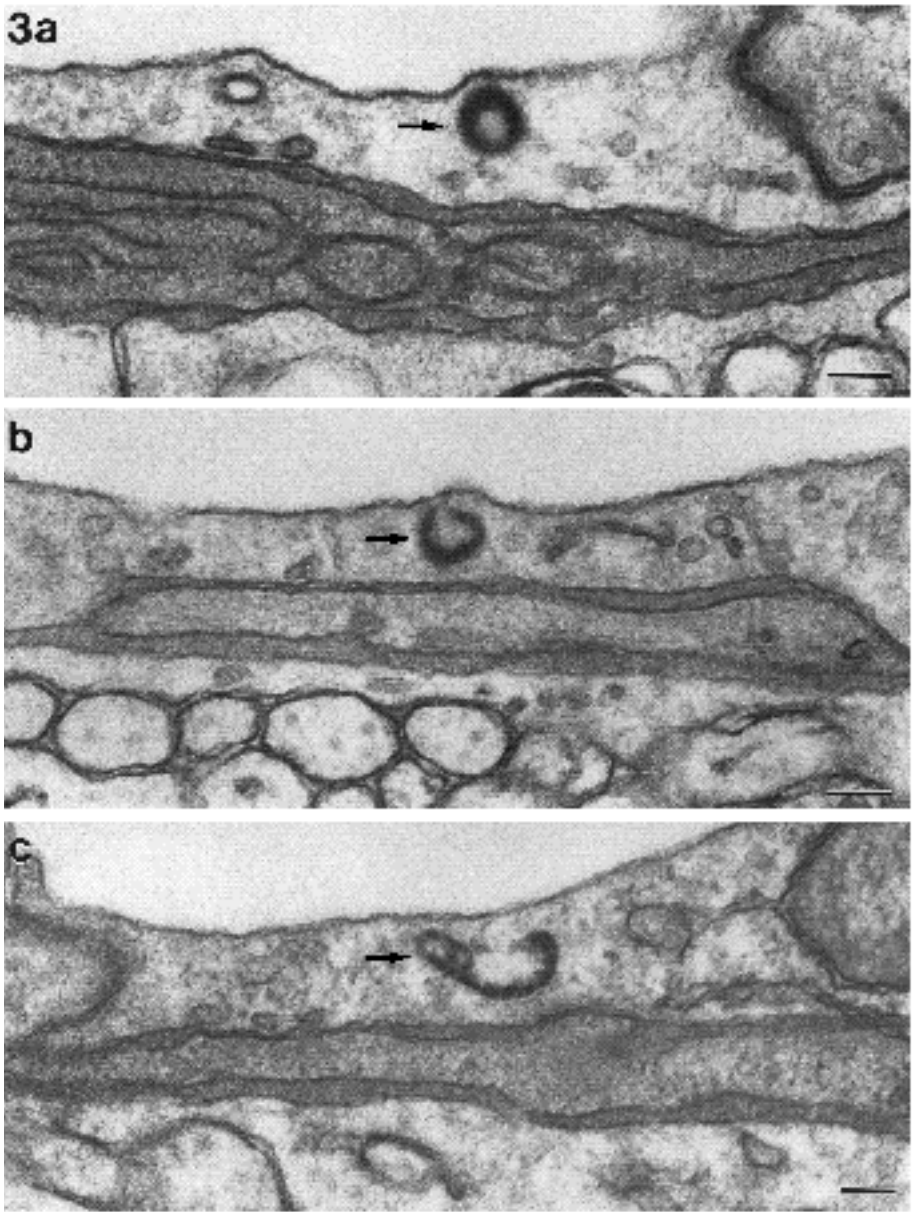

Fig 3. Localization (arrows) of Tf-HRP in rat brain capillaries after perfusion at 4˚C followed by a temperature shift to 37˚C for 2 min. Reaction product is localized in coated pits near the endothelial cells luminal surface (a) and (b), and in larger, non-clathrin-coated vesicles within the interior of the endothelium (c). Bars, 0.1 μm.

endothelial cells. Such structures, however, were rarely encountered after this time. Endothelial cells in brains that had been warmed for periods of time longer than 15 minutes only infrequently contained structures with any DAB reaction product, suggesting that nearly all internalized Tf-HRP had been processed and released by this time interval. When Tf-HRP was still present in these cells it was confined to cytoplasmic vesicles 100-200 nm in diameter. Multivesicular bodies were free of label at this time. Structures containing DAB reaction product did not appear fused to the basal membrane, nor was DAB reaction product identified in sub-endothelial locations including the interstitial space of neurons or glia. A quantitative summary of the labelled endothelial structures is presented in Table 1. Each DAB-containing organelle was assigned to one of the following categories: undifferentiated luminal membrane, uncoated pit, coated pit, early endosome, sorting endosome, recycling vesicle, undifferentiated antiluminal membrane, antiluminal coated vesicle, and antiluminal interstitial space.

In a second series of studies, rat brains were perfused with higher, more physiological concentrations of Tf-HRP (1 mg/ml) in oxygenated M199 at 30˚C for 15 minutes and then perfused with Tf-HRP-free buffer for 2-3 minutes prior to fixation and DAB cytochemistry. Regions of thalamus did not appear to be adversely affected morphologically by the protocol. Blood-brain barrier endothelial cells contained large amounts of DAB reaction product. In some endothelial cells most of the reaction product localized in 200-400 nm vesicles and tubules previously identified as sorting endosomes (Fig. 5a,b). Coated pits and vesicles as well as larger vesicles were also labelled in these experiments. 50-100 nm vesicles often appeared fused with the luminal membrane or were associated with the Golgi region, con-

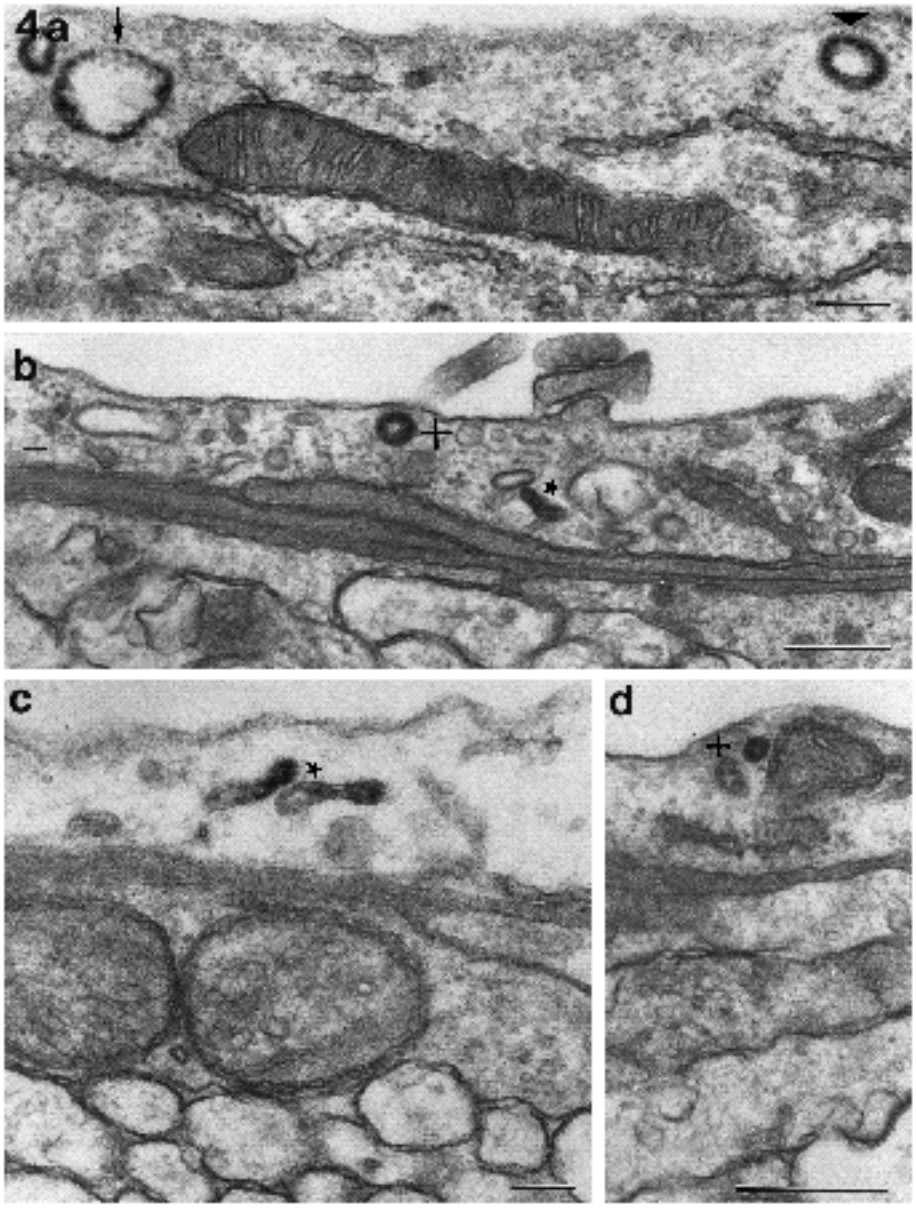

Fig. 4. Tf-HRP localization in rat brain capillaries after perfusion at 4˚C followed by a temperature shift to 37˚C for 5 min. Reaction product is localized in 250-500 nm vesicles identified as sorting endosomes (a, arrow), as well as coated vesicles (arrowhead). Also labelled are smaller tubular (stars) and vesicular (cross) structures (b,c), often in close proximity to the luminal membrane (d). Bars, 0.1 μm.

sistent with the recycling pathway described above (Fig. 5c). DAB reaction product was not found in the abluminal interstitial space or in endocytic vesicles of neurons or glia.

Thus, ultrastructural analyses of rat brains subjected to both types of Tf-HRP perfusions indicate that the major intracellular pathway followed by transferrin in brain endothelial cells is a pathway very similar to that described for other polarized epithelial cells. This pathway includes endocytosis by clathrin-coated vesicles at one cell pole, endosomal processing and recycling of receptor-ligand complexes back to the original membrane domain.

Washout analysis of transferrin perfused through brain endothelial cells

^{125}I-Tf readily bound to endothelial cell transferrin receptors after a 15 minute perfusion in the cold, and this binding was reduced by 80% when 1 mg/ml native rat transferrin was included in the perfusate. Consistent with the morphological studies, perfusion with warm buffer led to a rapid decrease in the amount of radioactivity remaining in the brain. The results of these studies are summarized in Fig. 6. It should be noted that after 30 minutes of warming approximately 30% of the maximal bound radioactivity remained associated with the brain and most likely represented ^{125}I-Tf in endothelial cells that had not yet been recycled and released or possibly transcytosed. A smaller portion (20%) of the counts remaining associated with the brains after 30 minutes of warming represents nonspecific binding. When the amount of nonspecific binding, determined from competition experiments, is subtracted from the radioactivity remaining after a 30 minute warming period, the percentage of counts remaining is only 10% of maximal binding. These results suggest that the major intracellular pathway for transferrin in brain endothelium involves recycling back to the luminal membrane and is consistent with the morphological observations.

Table 1. The distribution of transferrin-HRP in brain capillary endothelium[1]

	Time at 37˚C (minutes)				
Structure	0	2	5	10	15
Luminal membrane	21	4	2	2	3
Uncoated luminal pits	0	0	0	0	0
Coated luminal pits	79	18	2	0	0
Coated vesicles	0	61	10	0	0
Early endosomes	0	10	10	0	0
Sorting endosomes	0	0	42	18	8
Recycling vesicles	0	0	26	65	79
Antiluminal membrane	0	0	0	0	0
Antiluminal pits	0	0	0	0	0

Reaction product overlying the identified structures represents the average number of such positive structures per experimental animal. The s.e.m. among the animals was within 10%.

Immunolocalization of transferrin receptors in the rat brain

Indirect immunoperoxidase cytochemistry was done on frozen sections of rat brain using MRC OX-26, a cytoplasmic domain specific monoclonal antibody, as the primary antibody. A mouse anti-fibronectin monoclonal antibody was used as the primary antibody in a negative control.

Ultrastructural examination of tissue sections processed as described above revealed the presence of DAB reaction product exclusively on the luminal membrane of the brain endothelial cells (Fig. 7a,b). The labelling on the luminal membrane varied in intensity, with heavy labelling present over coated pits. In some capillary profiles reaction product was found only over luminal coated pits (Fig. 7c). In these experiments the tissue sections were not permeabilized with detergent prior to immunocytochemistry, so

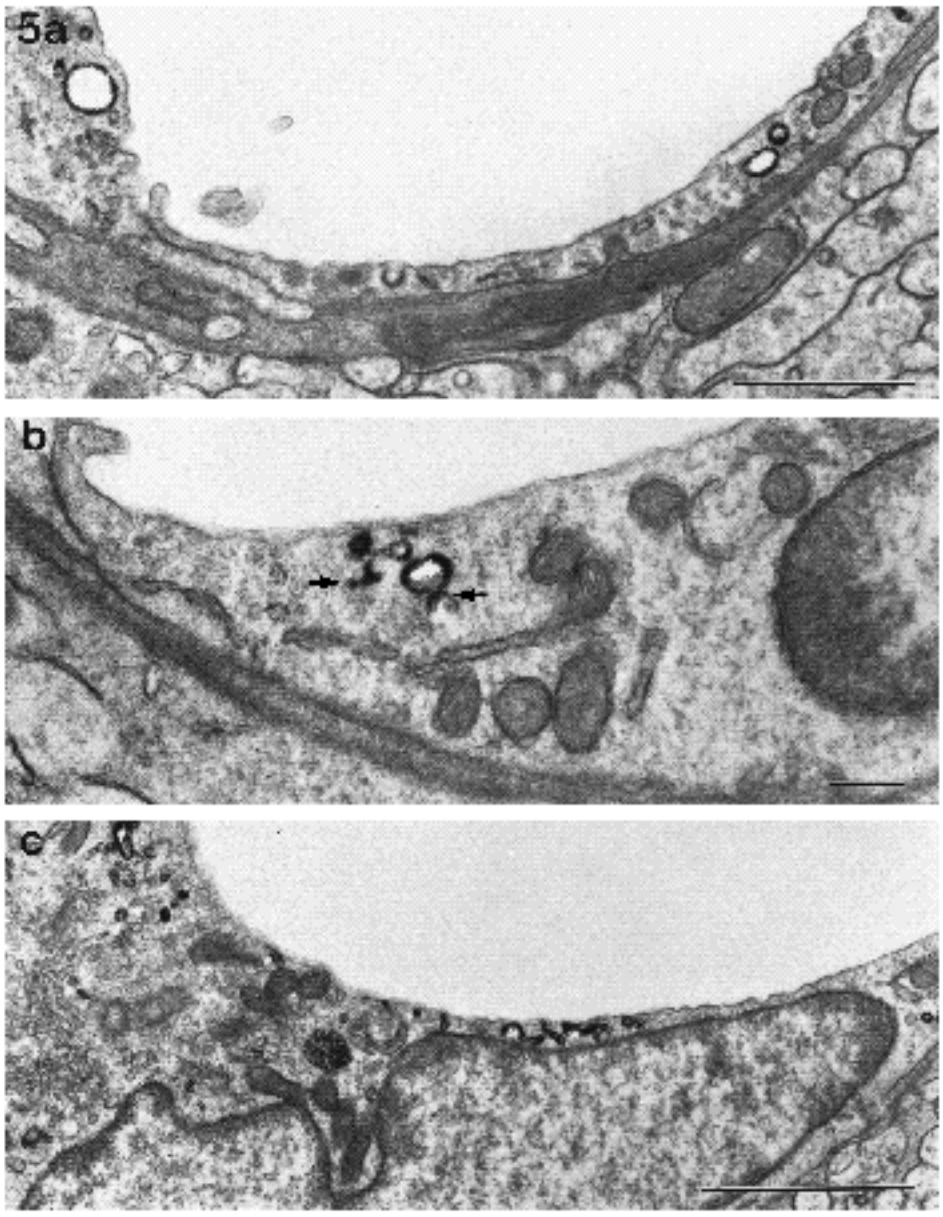

Fig. 5. Tf-HRP localization in rat brain capillaries after perfusion at 30˚C for 15 min. (a) Survey electron micrograph demonstrating large numbers of labeled vesicles and tubular elements; (b) the labeled structures identified as recycling endosomes (arrows) are preferentially associated with the luminal membranes. Bars, (a) and (b), 0.1 μm. In cells in which perfusion was carried out for longer than 15 min, reaction product appeared as in (c) and was confined to 100-200 nm cytoplasmic structures, also near the luminal membrane. Bar, 1.0 μm.

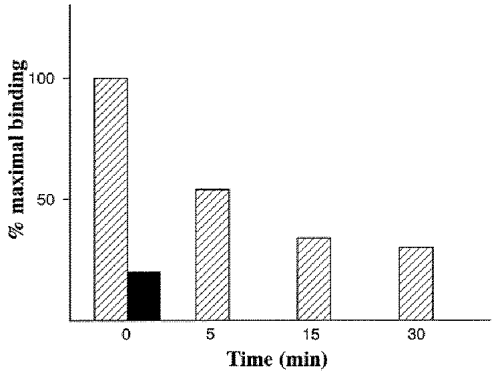

Fig. 6. Washout kinetics of ^{125}I-transferrin perfused into the rat brian for 15 min at 4˚C, followed by continued perfusion at 37˚C for the times indicated. Solid bar at time = 0 represents binding in the presence of excess (1 mg/ml) native transferrin. Brain radioactivity was determined by gamma counting. Results represent the average of three separate determinations.

intracellular vesicles containing the epitope which is recognized by the antibody were not exposed. Thus no intracellular reaction product was expected or observed, although the procedure should have allowed detection of transferrin receptor in the plane of the plasma membrane in either plasma membrane domain. DAB reaction product was notably absent from the basal membrane and areas of lateral intercellular contact between adjacent endothelial cells. A similar labelling in coated pit areas was observed when the secondary antibody was Protein A-collodial gold (Fig. 7d). No labelling was noted on the surface membranes of neurons and glia nor were coated pits and vesicles of these cells labelled. In control experiments the luminal membranes of brain endothelial cells were negative with respect to DAB reaction product (Fig. 7e).

DISCUSSION

In this study we have utilized morphological and biochemical techniques to characterize the endocytic pathway of transferrin in BBB endothelial cells. The results of these experiments support the notion that the major endocytic pathway of transferrin in cerebral endothelial cells is very similar to that observed in polarized epithelial cells (Fuller and Simons, 1986; Hughson and Hopkins, 1990). We have morphologically demonstrated that Tf-HRP specifically interacts with transferrin receptors on the luminal membrane of the endothelial cells and the resulting receptor-ligand complexes are subsequently bound and internalized

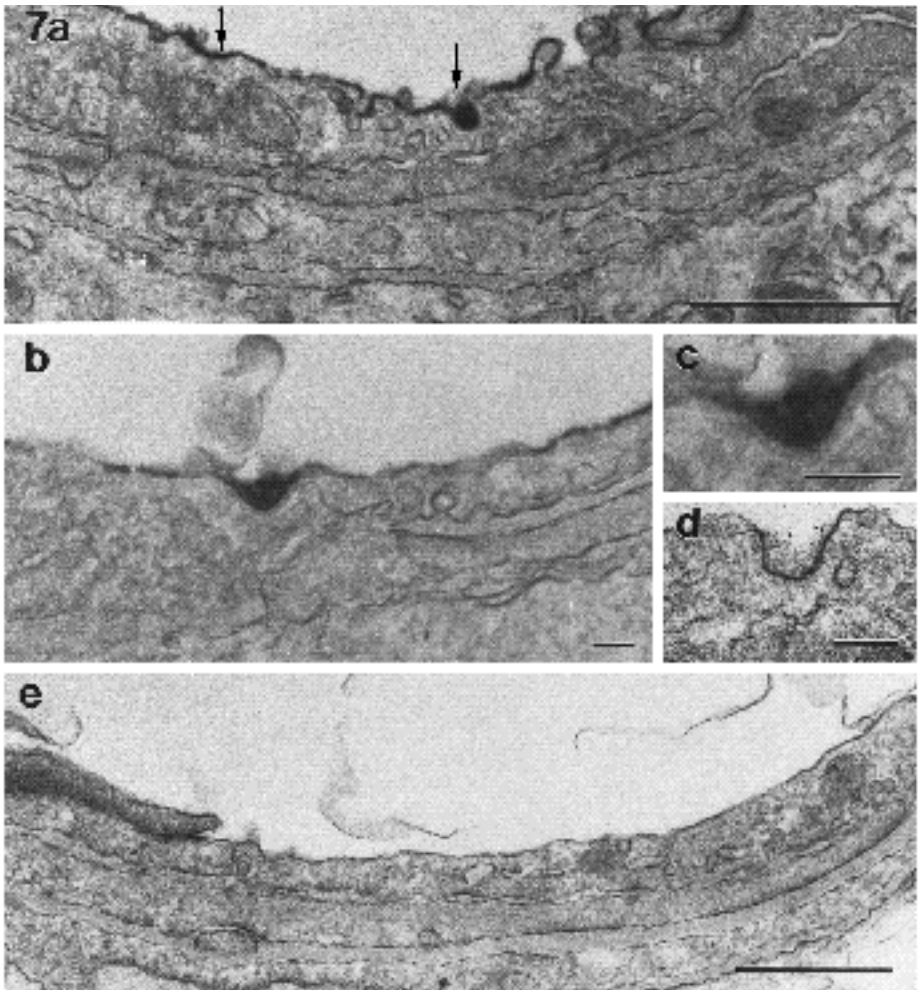

Fig. 7. Immunocytochemical demonstration of rat tranferrin receptor in rat brain endothelium. Receptor was localized by HRP immunostaining (a,b,c). In (a) reaction product appears over most of the plasma membrane, but is concentrated over putative coated pit areas (arrows). Bar, 1.0 μm. At higher magnification (b,c) the preferential staining over nascent coated pits is observed. Immunogold labelling (d) also detected the receptor in coated pit regions. Bars (b-d), 0.1 μm. Control (e), incubated without the OX-26 primary antibody. Bar, 1.0 μm.

in coated pits and vesicles, respectively. We have also shown that in brain endothelium, internalized Tf-HRP migrates through early endosomes, in which iron presumably dissociates from transferrin, and that apotransferrin-receptor complexes are sorted into recycling endosomes prior to release from the cell at the luminal membrane. The results of the ^{125}I-transferrin perfusions were also consistent with a major recycling pathway. Our results support the data reported by Taylor and Morgan (1990), which showed that most internalized iron dissociates from transferrin in the brain endothelium and that transferrin is rapidly recycled to the blood. However, these results and those of others using biochemical methods (Fishman et al., 1987), suggest a minor transcytotic pathway, which we did not detect morphologically in our studies. This could reflect the insensitivity of the morphological technique or the different conditions utilized among these studies. Our biochemical evidence (Fig. 6) suggests that as much as 10% of the specifically bound HPR-Tf is not retroendocytosed, but remains associated with the perfused brains. However, the ligand may attain access to the brain parenchyma in those regions where the BBB is not present. Thus a direct comparison of morphological data from a discreet region of the BBB and biochemical evidence from whole perfused brains or homogenates may not be appropriate.

In the temperature-shift experiments, Tf-HRP bound to endothelial cell receptors during a 15 minute perfusion at 4˚C. The concentration of Tf-HRP in the perfusate was 100 μg/ml, a concentration that is 20-40 times lower than is present in human serum. Because of these conditions, it is likely that much lower levels of transferrin were bound and internalized in these experiments than occur in vivo. However, we also failed to find evidence of transcytosis in the perfusions designed to analyze the transferrin pathway under conditions approaching steady state. In these studies a more physiological concentration of transferrin (1 mg/ml Tf-HRP; transferrin concentration 667 μg/ml) was used in order to reflect more closely in vivo conditions. If transferrin transcytosis were a relatively slow process requiring significantly more than 15 minutes to reach steady state, this would explain our failure to document a transcytotic pathway in brain endothelium. Transcytosis of polymeric IgA across hepatocytes in vivo initially takes 20 minutes after intravenous injection of the probe (Hoppe et al., 1985), although other reports suggest that the time required for transcytosis of ^{125}I-IgA across hepatocytes is about 10 minutes (Renston et al., 1980; Risau et al., 1990). In our system, perfusions longer than 15 minutes may result in unacceptable levels of hypoxic degeneration of brain elements as well as focal breakdown of the blood-brain barrier. Such damage, however, would presumably facilitate the appearance of HRP reaction product in the brain parenchyma, a phenomenon we did not observe.

Transcytosis of transferrin, therefore, could result either from a novel transcytotic pathway or from endosomal missorting in the recycling pathway. In polarized MDCK cells it has been reported that the accuracy of endosomal sorting of transferrin receptors to the proper membrane domain is greater than 99% during a single round of endocytosis (Fuller and Simons, 1986). The immunocytochemical localization of transferrin receptors presented here demonstrates that this receptor is highly polarized in cerebral endothelial cells, suggesting that in this cell type endosomal sorting may also be highly accurate. However, if transcytosis did occur as a result of endosomal missorting, iron would not necessarily accompany transferrin across the blood-brain barrier. In this study we have demonstrated that a large fraction of internalized transferrin is directed to endosomal subtypes which in other cells are acidic, leading to dissociation of iron from the carrier molecule; thus an additional mechanism would be required to facilitate the movement of this unloaded iron from the endothelium to the cells in the brain.

A potentially relevant model for a transferrin-iron transport system may be the trophoblastic cells of the placenta. The trophoblasts form the epithelial barrier that separates the maternal and fetal circulatory system, and developing organisms require large amounts of iron but the details of how iron is supplied are presently unknown, except it is clear that the placental barrier prevents maternal transferrin from reaching fetal tissues (Contractor and Eaton, 1986). To accommodate the fetal iron requirement, trophoblasts express large numbers of transferrin receptors which are active in the endocytosis of maternal transferrin. Trophoblasts, like blood-brain barrier endothelial cells, express these receptors on their apical membrane domain (Parmley et al., 1985). BeWo cells, a choriocarcinoma cell line of trophoblastic origin, have been shown to metabolize iron by a unique mechanism, capable of facilitating trans-placental iron transport in situ (van der Ende et al., 1987). These cells internalize iron by receptor-mediated endocytosis of transferrin. Iron dissociates from transferrin in acidic intracellular compartments and the newly released iron crosses the endosomal membrane and complexes with cytosolic ferritin by a poorly understood mechanism. BeWo cells are unusual in that much of the accumulated iron is subsequently released from the cell into the extracellular medium. This is in contrast to other cells such as HeLa cells, which release accumulated iron associated with ferritan only 5-10% as efficiently.

Applying the BeWo model to iron transport across the blood-brain barrier, we would propose that iron is endocytosed as a complex with transferrin in coated pits on the blood side of the brain capillaries. Iron dissociates from transferrin in endothelial endosomes, and some fraction of the liberated iron is released from the abluminal membrane by an unknown mechanism. Subsequent to this event, iron would complex with a transferrin-like molecule present in the interstitial space in the brain for delivery to neurons and glia. Several studies have demonstrated that transferrin is present in the brain. Connor and Fine (1986, 1987) have localized transferrin in different types of oligodendroglia by immunocytochemistry, while others have shown that transferrin is abundant in developing neurons in the rat (Dion et al., 1988). In these reports it is unclear whether the transferrin localized in these cells is derived from the blood or is synthesized in the brain. Transferrin mRNA has been localized in both oligodendrocytes (Bloch et al., 1985) and the epithelial cells of the choroid plexus (Bloch et al., 1987). Consistent with the mechanism we propose, transferrin receptors have been shown to be present on certain neurons

and reactive astrocytes where they presumably mediate iron uptake (Graeber et al., 1989; Orita et al., 1990).

Although the results reported in this study are consistent with a major retroendocytotic pathway of transferrrin through blood-brain barrier endothelium, we cannot completely rule out the possibility that in vivo transferrin transport still occurs by a mechanism that delivers iron still loaded to transferrin at the abluminal interstitial space. In this event, the conditions that would lead to the dissociation of transferrin from its receptor on the endothelium's abluminal membrane as well as the ultimate fate of transferrin in the brain are unknown. The brain lacks lymphatics so elimination of this protein from the brain would require the transport of apotransferrin back across the blood-brain barrier or the targeting of transferrin to lysosomes for degradation. Bidirectional transport of transferrin across the blood-brain barrier has previously been described (Banks et al., 1988). In this study, ^{125}I-transferrin was injected intraventricularly and brain-associated radioactivity was followed over time. The decrease in brain-associated radioactivity noted in this study might be due to the movement of transferrin from the ventricles into the leaky, non-blood-brain barrier type capillaries that supply the circumventricular organs in the CNS. Thus, the efflux of this protein may occur by non-specific migration through these leaky capillaries and not by specific apotransferrin receptors on the endothelium's abluminal membrane.

While the pathway of transferrin endocytosis and recycling in cerebral endothelium is similar in other epithelial cells, it differs in at least one important aspect. Like all epithelium, cerebral endothelial cells exhibit striking polarization of many membrane proteins. Ultrastructural cytochemical studies have shown that Na^+,K^+-ATPase is present only on the basal membrane while alkaline phosphatase is a marker for the apical or luminal membrane (Beck et al., 1986; Betz et al., 1980). This reflects the arrangement of these marker enzymes in epithelium (Ernst and Schreiber, 1981; Robinson and Karnovsky, 1983). In well characterized epithelial cells including MDCK, Caco2 and hepatocytes, transferrin receptors and other membrane proteins involved in nutrient uptake (e.g. LDL receptor) are present on the basolateral membrane where they have access to molecules carried in the blood (Fuller and Simons, 1986; Hughson and Hopkins, 1990; Levine and Woods, 1990). However, in this study, using the same fixation protocols and antibodies, we have shown that the transferrin receptors of cerebral endothelial cell are polarized and are located on the luminal membrane. For these experiments, postembedding labeling conditions were used in an attempt to detect receptors on both membrane domains, if present. Previous studies localizing OX-26 to brain capillaries have relied on light level microscopy which could not directly address the issue of polarity. Why transferrin receptors are sorted to different membrane domains in different types of polarized cells is unknown. Recent studies have investigated the general mechanism by which membrane proteins are sorted to specific membrane domains in epithelium (Le Bivic et al., 1990; Lisanti et al., 1989). In MDCK cells, apical membrane proteins possess sorting signals that are recognized in the *trans*-Golgi network which selectively sorts these membrane proteins to the apical membrane (reviewed by Simons and Wandinger-Ness, 1990). Basolateral membrane proteins, like the transferrin receptor, may move by default to the basolateral membrane along the constitutive secretory pathway (reviewed by Pfeffer and Rothman, 1987). In endothelial cells, the polarity of the regulated and constitutive pathways may have the opposite orientation. Consistent with this hypothesis, Sporn et al. (1989) have shown that the regulated secretion of von Willebrand factor in cultured endothelial cells is almost entirely basolateral. This is in contrast to regulated protein secretion in many types of secretory epithelia where these proteins are secreted at the apical membrane (reviewed by Burgess and Kelly, 1987; Kelly, 1985). Bacallao et al. (1989) have described changes in the subcellular organization of MDCK cells that accompany the formation of a phenotypically polarized epithelium. Two characteristics of polarized MDCK cells reported here that may be important in the regulated secretory process are the orientation of microtubules and the position of the Golgi complex. In polarized MDCK cells, longitudinal microtuble bundles stretch from the apical to the basolateral pole and are oriented with their minus ends at the apical cell pole and their plus ends at the cell base. Also, the Golgi complex was supra-nuclear in polarized MDCK cells. Typically in epithelium, the *trans*-Golgi element faces the apical membrane. In blood-brain barrier endothelial cells it is not uncommon for the *trans*-Golgi elements to face the abluminal membrane (unpublished observation). This may reflect an alternative orientation of the microtubule cytoskeleton in endothelium. If endothelial cells contain longitudinal microtubule bundles positioned with the opposite orientation as exhibited in epithelium, that is, plus ends apical and minus ends basolateral, mechanisms explaining the differences in the polarity of the regulated and constitutive pathways could be hypothesized and potentially tested. The resolution of these questions awaits the development of a brain endothelium culture system that retains and maintains a fully differentiated phenotype.

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