New systems for delivery of drugs to the brain in neurological disease

Eain M Cornford and Marcia E Cornford

The restricted or regulated entry of most blood-borne substances into the brain has been recognised for more than a century. The blood-brain barrier (BBB)-shielding function provided by endothelial cells is important in the treatment of neurological diseases because this exclusion of foreign substances also restricts entry of many potentially therapeutic agents into the brain. The recent identification of several neuroactive proteins of potential therapeutic value has highlighted the crucial need for effective and safe transcapillary delivery methods to the brain. One promising method is delivery through brain capillaries by augmentation of pinocytotic vesicles; delivery systems that use this cellular mechanism are in development. Recent investigations in animal models show that large molecules of neurotherapeutic potential can be conjugated to peptidomimetic ligands, which bind to selected peptide receptors, and are then internalised and transported in small vesicles across the cytoplasmic brain capillary barrier. These conjugates have been shown to remain functionally active and effective in animal models of neurological disease.

Lancet Neurology 2002; **1:** 306–15

The fact that dye injected intravenously into animals stains all body tissues except the brain has been known for more than a century; this observation shows that the brain capillaries prevent molecules from entering the intact central nervous tissue. Thus, the concept of the blood-brain barrier (BBB) was established. The BBB is a result of the endothelial cells of normal brain capillaries, which have many unique properties.[1–6] Endothelial cells of brain capillaries have fewer pinocytotic vesicles[2] and more mitochondria than those of capillaries elsewhere in the body, and the capillaries themselves lack fenestrations and interendothelial passages (figure 1). Continuous zones of plasma membrane adherence form tight junctions that prevent passage between brain endothelial cells. Movement from blood to brain therefore requires transcellular passage, whereas fenestrations and clefts make peripheral-tissue capillaries much more permeable.[1] Not unexpectedly, the most promising human gene therapies (eg, those for haemophilia and cardiovascular diseases) are characterised by the ease of vascular access to target cells.[7] Transcellular drug delivery to the brain is apparently further complicated by the presence of several cytoplasmic enzymes, such as γ-glutamyl transpeptidase, alkaline phosphatase, and aromatic-acid decarboxylase, which are more abundant in, or unique to, the endothelial cells of brain capillaries. These proteins form an enzymatic barrier within the anatomical structure.[4,5] Drugs of large molecular size are known not to gain access to the brain, but what is not widely recognised is that more than 98% of drugs of smaller molecular size do not cross the BBB.[8]

There are three different groups of endogenous transport systems in brain capillaries: carrier-mediated transporter systems, active-efflux transporters, and receptor-mediated transcytosis systems. Carrier-mediated transporter proteins, present on both luminal and abluminal membranes, regulate brain entry of plasma-borne metabolites—such as glucose, amino acids, organic acids, choline, and purines—down a concentration gradient. Some of these transporters have unequal expression on the luminal and abluminal membranes:[1,5] for example, sodium potassium ATPase and the sodium-dependent neutral-amino-acid transporter are found in higher densities on the abluminal membrane, whereas γ-glutamyl transpeptidase is more abundant on the luminal membrane. This polarity is thought to facilitate bidirectional regulation of the entry and exit of many different plasma-borne molecules. Active-efflux systems include transporters such as P-glycoprotein,[8] and mediate the energy-dependent brain-to-blood efflux of various small molecules against a concentration gradient. The receptor-mediated trancytosis systems, such as the BBB transferrin receptor and the insulin receptor, mediate the bidirectional transit of large peptides between blood and brain.[2,8] Studies of brain-vascular genomics (which identify gene families that are differentially expressed on the BBB) have identified several previously unrecognised transporter and receptor proteins, which include some with as yet undefined functions.[9,10]

Delivery of compounds across the BBB: strategies and methods

The first attempts to overcome difficulties in delivery of drugs to the brain were directed towards improving lipid-mediated entry. The main factors that limit the penetration of lipophilic membranes by any molecule are its molecular weight and its ability to form hydrogen bonds and so bind to circulating plasma proteins. An alternative strategy, applicable to certain drugs, is to inhibit brain exit. For example, the protease inhibitors used in the treatment of AIDS are substrates for P-glycoprotein,[11] and the nucleoside analogue zimovudine and the reverse-transcriptase inhibitor lamivudine are substrates for other active-efflux transporter systems in the

EMC is at the Department of Neurology and the Brain Research Institute, UCLA, and the Department of Veterans' Affairs, Los Angeles. MEC is at the Department of Pathology and Laboratory Medicine, UCLA, Los Angeles, and the Department of Pathology Harbor-UCLA Medical Center, Torrance, CA, USA.

Correspondence. Dr Eain M Cornford, Department of Neurology, UCLA School of Medicine, 710 Westwood Boulevard, Los Angeles, CA 90095–1769, USA. Tel +1 310 268 4426; fax +1 310 478 4538; email cornford@ucla.edu

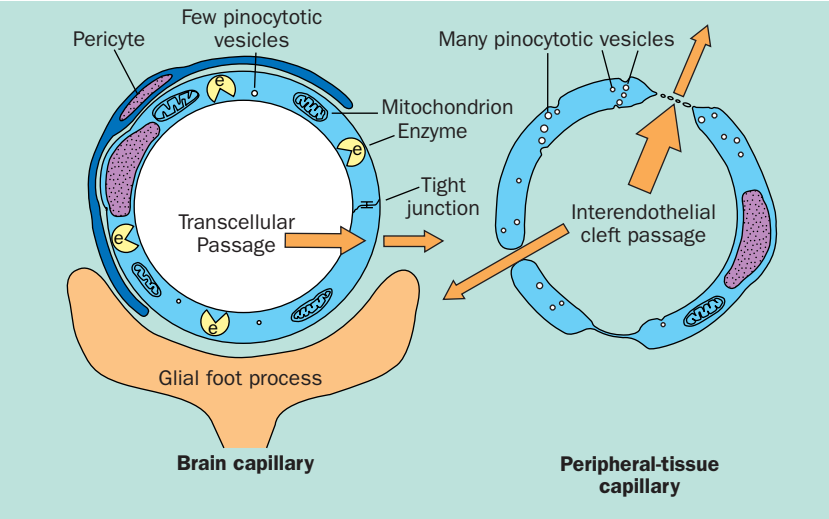

Figure 1. Brain capillaries differ from those in peripheral tissue. The acellular basal lamina encircles the endothelial and pericyte cells and separates them from the glial foot process. Endothelial cytoplasm is of uniform thickness, lacks fenestrations, and has few pinocytotic vesicles. Fenestrations and clefts present in typical peripheral-tissue capillaries make them much more permeable. The pericyte serves as the first line of defence if brain-endothelial-cell integrity is compromised. High-resistance tight junctions seal the cell-to-cell contacts between adjacent endothelial cells. Unique and concentrated enzymes in the endothelial cells and glial processes add to the barrier effect.

BBB.[12,13] Pardridge[8] has proposed the development of efflux-transporter inhibitors, which would increase brain concentration of protease inhibitors, zidovudine, and lamivudine and thus improve drug therapy. This strategy of BBB-directed drug augmentation is already used when inhibitors of aromatic-acid decarboxylase are coadministered with levodopa to inhibit its metabolism.

With the recent advances in the understanding of how receptor-mediated transcytosis systems operate, the focus of brain drug delivery has switched to mechanisms that use the endogenous capillary receptors, since these systems promote membrane passage of specific large molecules. Pardridge[14] noted that the only endogenous peptides that cross the BBB to any appreciable extent (eg, insulin, insulin-like growth factor, and transferrin) use receptor-mediated endocytosis. Specific receptors facilitate the movement of these compounds across the luminal capillary membrane, through the cytoplasm, and across the abluminal membrane to the neuropil. This process involves binding of the receptor and peptide at the luminal membrane of the BBB, formation of a plasmalemmal vesicle, translocation of the receptor-peptide complex through the cytoplasm, and dissociation of the peptide from the receptor on the external surface of the abluminal membrane. Enzymatic degradation of the drug does not occur within the endothelial cell, because the receptor-bound ligands are contained within membrane-sealed pinocytotic vesicles. Such receptors, which can transmit large molecules across the BBB (and tend to have similar transport properties when linked to potential neuropharmaceuticals), offer the promise of serving as useful "Trojan horses" in the development of new transport vehicles to facilitate BBB passage.[2] Similarly, modified proteins such as cationised albumin also cross the brain capillary via a transcytosis mechanism, but no specific receptor for this protein has yet been identified. An absorptive-mediated transport system is thought to be present, which would also be useful as a drug transport vehicle.[8]

BBB capillaries are fully capable of transcytosing specific proteins, although some parts of this process may need further investigation.[15] Plasmalemmal vesicles are known to represent the "large pore" transcytosis system in microvascular endothelial cells.[16] Ultrastructural marker studies show that vesicles mediate albumin transcytosis in endothelial cells, and there is little, if any, passage of albumin via interendothelial junctions.[17] No brain-capillary receptor for the polysaccharide inulin has been identified, but receptors for insulin and cationised albumin have been characterised.[14] Although morphologically similar, populations of endothelial-cell vesicles in peripheral tissues are functionally diverse (ie, those that contain inulin are functionally different from vesicles containing insulin and albumin.[18] Transferrin-recycling vesicles have also been distinguished from a second population of vesicles on the basis of different sorting functions.[19] In the BBB, endothelial vesicles from heterogeneous populations appear to be functionally defined on the basis of their plasmalemmal receptor proteins that determine the content of each vesicle. Restricted BBB penetration of some molecules may be due to regulated expression of specific receptors, rather than inhibition of the transcytosis mechanism in brain capillaries.[15] This restricted receptor density could presumably explain why myocardial and pulmonary capillary cells have 1000 and 100 pinocytotic vesicles per μm^3 of endothelial cytoplasm, respectively; by contrast, fewer than ten vesicles per μm^3 of cytoplasm are found in brain capillaries.[14]

Vesicular transcytosis mechanisms may also be important for the understanding of differences in the permeability of the BBB around tumours. Selective opening of the BBB at brain tumours, without modification of the adjacent capillaries, has been achieved by the administration of leukotrienes and vasoactive modulators such as bradykinin. Several cellular mechanisms seem to contribute to raise the permeability locally, and these treatments cause increases in the number and size of endothelial-cell vesicles within the brain tumour capillaries but not in the capillaries of the normal contralateral hemisphere.[20]

Vector-mediated transport across the BBB

Peptide receptors selectively bind their high-molecular-weight ligands and achieve BBB transport by receptor-mediated endocytosis at the luminal capillary membrane, followed by abluminal-membrane exocytosis into the brain interstitium. As long ago as 1987, the addition of non-transportable drugs to peptide vectors was shown not to affect vector transcytosis through the brain endothelium, and brain delivery was

accomplished by the formation of chimeric peptide neuropharmaceuticals.[21] The chimeric peptide model involved synthetic coupling of the non-transportable peptide (eg, β-endorphin) to a transportable vector such as cationised albumin.[22–25] Other endogenous ligands, such as transferrin[26] and insulin fragments, have also been successfully used to deliver peptides across the BBB in animal studies.[27] Brain capillaries express several other known receptors—including those for insulin-like growth factor, leptin, low-density lipoprotein, and lectins—that transport these endogenous peptides through the BBB and could also potentially deliver conjugated drugs.[14]

Fusion protein vectors

Conjugation of the desired drug to a selected vector in a reproducible, high-efficiency system is essential for therapeutic purposes. This need resulted in the development of monoclonal antibody (MAb) fusion proteins. A transport vector is directly coupled to the selected non-transportable drug by generation of a fusion protein from customised DNA. Non-transportable specific antigen-binding monoclonal antibodies, such as IgG3, have been bonded to a transport vector (such as transferrin or insulin-like growth factor) by linking the DNA sequences coding for both molecules. Shin and co-workers[28] showed that these conjugates not only were capable of vector-mediated BBB transit, but also retained such functions as complement-mediated lysis characteristic of the IgG molecules. Other fusion proteins such as transferrin–nerve growth factor have also been produced for delivery of neurotrophins to the CNS.[29]

MAb vectors

Several studies have investigated drug delivery to cells and tissues with transferrin as a delivery vector.[30–32] The observation that OX26, a mouse MAb to the rat transferrin receptor, could attach to the endogenous transferrin-receptor-mediated transcytosis mechanism to cross the abluminal membrane and deliver large molecules into the brain[14,33] has stimulated further studies of MAb vectors.[34,35] Transferrin was localised in isolated capillaries (in vitro) by use of confocal microscopy, and radiolabelled transferrin uptake in rat brain was visualised with thaw-mount autoradiography.[36] Electron microscopy of brain capillaries has shown that immunogold-labelled OX26 binds to the luminal membrane, apparently enters the capillary cytoplasm, and then undergoes exocytosis into the brain.[37] Some researchers advocate the use of transferrin as a delivery vector in comparative studies,[26] whereas others suggest that transferrin and OX26 are equally effective brain delivery vehicles.[38] Importantly, the human brain-capillary transferrin receptor is 50% saturated by 5 nmol/L transferrin, whereas the normal plasma concentration is 25 μmol/L. Therefore this receptor is normally fully saturated,[14] and competition for receptor binding could significantly reduce the binding and penetration of transferrin-linked drugs. By contrast, OX26 binds to a different site on the receptor, is unaffected by endogenous transferrin, and is potentially more efficient.

Large peptides, such as brain-derived neurotrophic factor[39] and nerve growth factor, linked to OX26 have been vector-delivered to the brain. Since neuronal growth and regeneration may require only trace quantities of this protein, this biotechnology has potential clinical use in the treatment of neuronal degeneration or the stimulation of brain repair. Intravenously administered OX26–nerve-growth-factor also stimulates the survival of fetal-brain cholinergic neurons grafted into the anterior chamber of the eye.[40] Another application of the technology is its use in the transport of antisense oligodeoxynucleotides, designed to regulate the expression of a specific gene. These potential neuropharmaceuticals have also been conjugated to streptavidin–OX26 for BBB transit.[41] Pardridge and colleagues[42] have shown that transport across the BBB of a monobiotinylated polyamide nucleic acid conjugated to the streptavidin–OX26 vector is 28 times that of the unconjugated molecule. Factors such as metabolic stability, plasma-protein binding, and adequate cell-membrane transport can be overcome with OX26 conjugation to acheive brain delivery of these compounds. The potential applications of antisense-based therapeutics in brain tumours and Alzheimer's disease, for example, are discussed below.

Design and manipulation of vector systems

Linkage of the MAb vector to the drug makes use of the strong affinity of the protein avidin for the peptide biotin as commonly used in immunocytochemical antigen binding. In the original technique, avidin was conjugated to the MAb, which was attached to a biotin moiety and linked to the transportable drug by disulphide bonds. A biotinylated analogue of vasoactive intestinal peptide, coupled to an avidin–OX26 conjugate, was shown to raise cerebral blood flow in rats, but intracarotid administration was required.[34] To prevent rapid removal from the peripheral circulation, Wu and Pardridge[35] subsequently conjugated vasoactive intestinal peptide to streptavidin (neutral avidin) and OX26: this conjugate increased cerebral blood flow rate after intravenous administration, with attenuated side-effects in peripheral organs such as the salivary glands.

When peptide drugs are linked with much larger MAb proteins, steric hindrance of the neuroactive molecule by the MAb delivery system may also be a problem. Steric hindrance was shown when radiolabelled epidermal growth factor (EGF) was linked to OX26.[43] Separation of EGF and OX26 with a polyethylene-glycol linker was needed to acheive optimum binding of the conjugate to both the capillary transferrin receptor and the EGF receptor. Consequently, the design of many vector systems now includes a 200-atom polyethylene-glycol linker to distance the MAb transport vector from the non-transportable neurotherapeutic protein.

In describing various drug-vector linker strategies, Pardridge[37] noted that labile disulphide bonds can be used if cleavage of the delivery vector is desired. Vector-mediated neuropharmaceuticals must retain their activity through intravenous transport and transit across the BBB. Kang and colleagues[44] have shown that avidin–biotin disulphide bonds do not undergo cleavage during transcytosis through brain endothelial cells. Vector persistence is a theoretical problem. However, Zhang and Pardridge[45] showed that IgG molecules undergo a rapid brain-to-blood efflux, presumably via an Fc

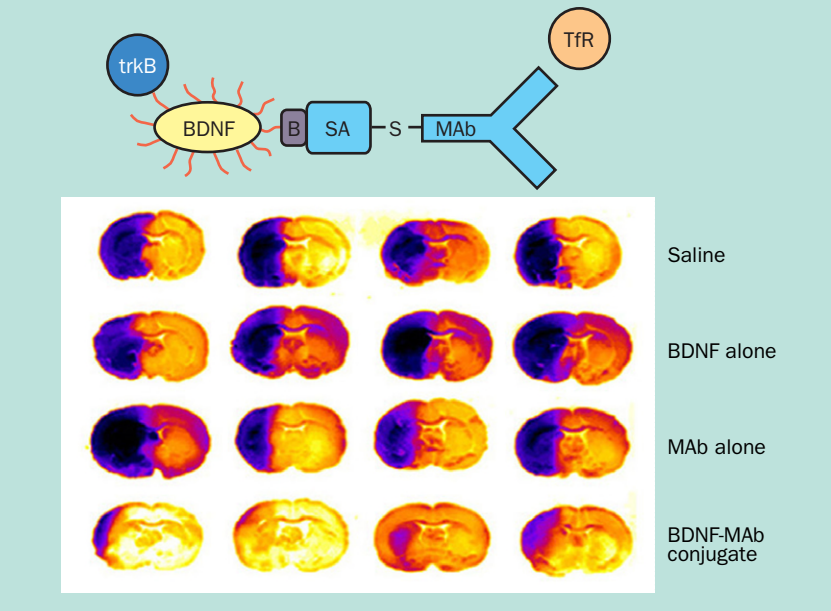

Figure 2. A delivery system for brain-derived neurotrophic factor (BDNF), which has been shown to limit infarct size in a rat model of regional brain ischaemia.[47,49] Top: the chimeric peptide is bifunctional, as the molecule binds to the neuronal trkB receptor and the transferrin receptor (TfR) on the BBB. The neurotrophic factor is coated in strands of polyethylene glycol, one of which contains a biotin moiety (B) to enable binding to streptavidin (SA), which is conjugated to the OX26 monoclonal antibody (MAb) through a stable thioether linker (-S-). Bottom: 2,3,5-triphenyl tetrazolium chloride stains are shown for 16 different rats, comprising four rows of different treatment groups. In the rats receiving BDNF-MAb conjugate, the infarct zone (purple) is 65% smaller than in controls. Reproduced with the permission of the American Medical Association.[8]

receptor that mediates removal (by reverse transcytosis) of immunoglobulins from the brain. Thus, even after multiple conjugate treatments, any significant brain accumulation of the MAb fragments seems unlikely.

Potential applications of chimeric vectors to achieve brain-targeted delivery

Experimental stroke studies

Treatment of cerebral ischaemia with brain-derived neurotrophic factor, which has been shown to have protective effects when administered intracerebrally,[37] is hampered because it does not cross the BBB. When brain-derived neurotrophic factor is linked to polyethylene glycol and joined to OX26, it can be successfully transported across the BBB. A very appealing feature of this strategy is that the biotin-linked therapeutic molecule could be linked to the streptavidin-MAb vector immediately before use at the bedside; owing to the very high affinity of avidin for biotin, there is instantaneous capture of the MAb vector. This technique has been applied only in studies of experimental stroke in rats. Rats were subjected to transient global ischaemia and then intravenously given buffer, brain-derived neurotrophic factor, or OX26 antibody; 7 days later there was a 70% loss of hippocampal neurons.[46] By contrast, there was no significant loss of neurons in rats treated with the neurotrophic factor (250 μg/kg) conjugated to OX26 immediately after the global ischaemic event compared with non-ischaemic controls.[46]

Similar protection was also confirmed in two subsequent studies of regional brain ischaemia in rats in which administration of the conjugate of brain-derived neurotrophic factor and MAb was given for 1–2 h after the ischaemic insult.[47] Remarkable neuroprotection[48] occurred in rats that were rendered ischaemic by occlusion of one middle cerebral artery for 24 h.[49] Coronal sections of rat brains showed that the size of the infarcted region was large and unchanged in control rats injected with saline, neurotrophic factor alone, or MAb alone (figure 2). By contrast, the infarcted region was 65% smaller in rats intravenously given 50 μg of the conjugate 1 h after reperfusion than in the controls. When treatment was delayed until 2 h after the insult, the infarct volume was only 30% smaller in the actively treated rats.[49]

The dose-response effect of the conjugate treatments was also examined in the same regional brain ischaemia model. With doses of 5 μg and 50 μg of brain-derived neurotrophic factor per rat, infarct volume was significantly smaller (43% and 65%, respectively) when the factor was administered as a conjugate. When two conjugate treatments were given at baseline and 3 h, 1 h and 4 h, or 2 h and 5 h after-insult, dual therapy was shown to extend the therapeutic window beyond 2 h.[49] The delayed administration of a neuroprotectant after stroke in human beings is important because, in an emergency-care situation, clinical neuroprotection measures might be possible only within this extended time-frame. Furthermore, the dual-therapy strategy could use brain-derived neurotrophic factor in combination with a second neurotrophic factor designed to act at a different stage of the ischaemia-induced apoptotic pathway.[49]

One such potential dual-therapy neurotrophin has already been defined. Song and co-workers[50] have recently shown that basic fibroblast growth factor (coupled to the OX26 delivery system) was associated with an 80% reduction in infarct size when administered immediately after insult and a 67% reduction when administered 1 h after regional ischaemia. Because erythropoetin, neurotrophin 3, transforming growth factor α, vascular EGF, and EGF have all been shown to convey neuroprotection after brain ischaemia; any one of these factors might also augment treatment with MAb-conjugated neurotrophic factor[49] or basic fibroblast growth factor.[50]

Since neuroprotection is possible only during the initial hours after-insult, when the integrity of the BBB in the stroke region is not yet disrupted, an important footnote is that all neurotrophin therapeutics must be conjugated to a brain delivery vector to be effective. Thus, animal-model stroke studies show that coupling of a previously identified neuroprotective protein to an effective brain delivery system,[47,49,50] in a clinically relevant time-frame, can produce a useful post-ischaemic therapy in human beings. Both the

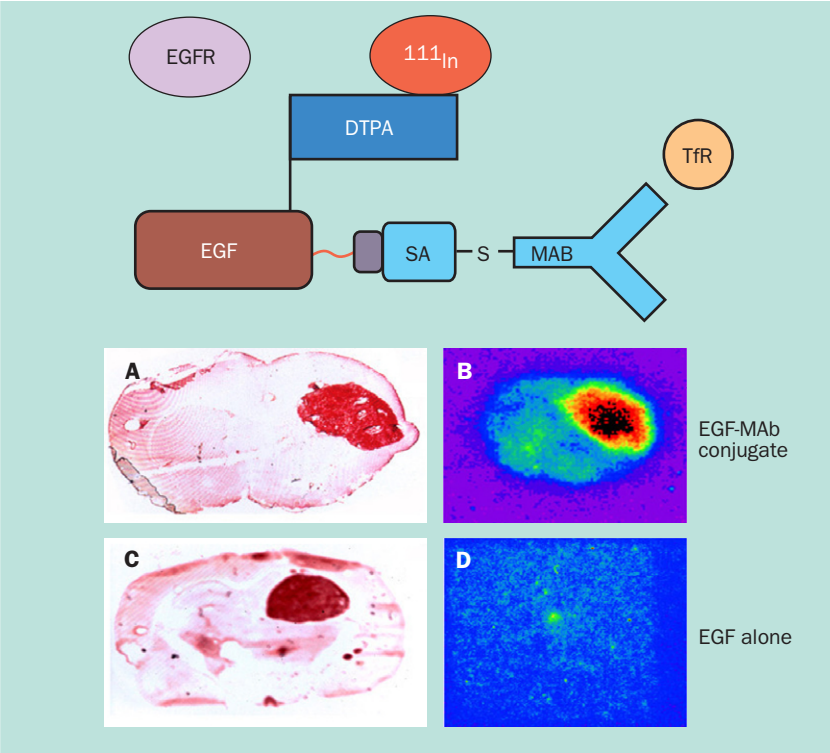

Figure 3. Brain tumours can be identified by imaging of overexpressed cellular proteins in vivo. Top: EGF chimeric peptide is radiolabelled with diethylenetriamine penta-acetic acid (DTPA) which chelates ^{111}In. EGF is attached to a biotinylated polyethylene-glycol linker, and this complex is captured by a conjugate of streptavidin (SA) and OX26. Bottom: light microscopy of autopsy sample shows experimental human U87 human gliomas implanted into nude rat brains (A and C). In rats given the conjugate, in vivo brain scans accurately image the tumour (B). There is no imaging of the brain tumour when the EGF peptide is administered alone (D). Reproduced with the permission of the American Medical Association.[8]

discovery of effective protective agents and the successful volume delivery of these agents to ischaemic brain are equally important in the quest for effective clinical stroke treatments.[48]

Experimental imaging: tumours and amyloid

Imaging of animal-model tumours with BBB delivery systems has also shown promise as a use of vector-mediated BBB penetration designed to identify either increased gene expression or overexpressed cellular proteins in tumours. One potential mechanism for imaging the altered gene expression characteristically seen in brain tumours is the use of peptide nucleic acids, which are most readily delivered to the CNS when conjugated to a streptavidin–OX26 delivery vector.[51] These antisense oligodeoxynucleotides hybridise to target nucleic acids in the brain tumour via mechanisms specific to the nucleic-acid sequence. Unlike other oligodeoxynucleotides, peptide nucleic acids are not susceptible to exonucleases or endonucleases, and they are stable in serum or cell culture. A carboxyl-terminal tyrosine or lysine residue can be readily added to the peptide nucleic acid to enable radiolabelling with iodine-125 or indium-111, respectively, for clinical imaging studies.[37] Shi and colleagues[52] have described the conjugation of an iodinated peptide nucleic acid with OX26 to the transferrin receptor, and its use in successful imaging of C6 gliomas in experimental rat tumours. Transport of the antisense imaging agent through two membrane systems (the BBB and the tumour-cell membrane) was achieved by OX26 targeting to the transferrin receptor, which is present on both endothelial-cell and tumour-cell membranes.[52]

The human insulin receptor is highly expressed throughout human brain tissues.[37] In high-grade human brain gliomas, the insulin receptor on capillaries of the normal BBB and barrier between blood and tumour can be immunocytochemically localised. A chimeric human insulin receptor MAb has been prepared[53] that may enable imaging of gene expression in the human brain[37] analogous to OX26 in rats. 63% of highly malignant human brain tumours (glioblastoma multiforme) overexpress the EGF receptor, hence, linkage of therapeutic agents to this tumour-specific receptor protein is a potentially useful target in the imaging of such tumours. Even very small human brain tumours might be imaged if discriminating peptide radiopharmaceuticals that targeted tumour-specific receptors could be developed.[54] This hypothesis led to the synthesis of an EGF peptide radiopharmaceutical, conjugated to a delivery system to permit delivery across the BBB. EGF labelled with ^{111}In, which was linked in turn to polyethylene-glycol–biotin, with a streptavidin linkage to OX26 (figure 3). The animal tumour model used human U87 glioma cells (which express functional EGF receptors), implanted into nude rat brains.[54]

Neuroimaging in rats bearing U87 human gliomas in vivo accurately located large and small brain tumours with the EGF-conjugated delivery system, because the EGF-receptor density is much greater in the tumour than normal brain (figure 3). Conversely, radiolabelled EGF given without the vector did not target or identify even large tumours.[54] Because of the high expression of the EGF receptor in normal liver, a single intravenous injection of unlabelled EGF was also administered to saturate hepatic uptake of the EGF chimeric peptide and promote brain delivery.[55] Therefore, BBB delivery systems may not only be useful for drug delivery, but also have a potential use in neuroimaging for diagnosis of tumours.[52,54,55]

The severity of dementia in Alzheimer's disease correlates with the progressive deposition of a unique immunogenic extracellular amyloid protein. This process provides another potential application of vector-conjugated brain delivery methods directed at non-invasive imaging of this protein in patients with suspected Alzheimer's disease.[56] This technique could enable early treatment of individuals by diagnosis before symptoms are advanced.[2]

Brain-directed immunoliposomes

A related strategy for achieving transit of neuropharmaceuticals across the BBB uses the attachment of immunoreactive moieties to liposomes. The formation of immunoliposomes designed to target specific tissues has been known for two decades.[57–59] However, the cellular barrier formed by tight junctions between brain endothelial cells was assumed to exclude all plasma-borne liposomes,[14] even small (diameter 50–80 nm) unilamellar vesicles.[37] Perhaps the most striking advance in BBB transit techniques during the past few years is the demonstration that immunoliposomes can be used for brain delivery of drugs, as well as other neuropharmaceuticals such as oligonucleotides.[37]

The potential effect of this approach is enormous, because it offers a mechanism by which any molecule that can be encapsulated in a liposome can be directed to the brain. Liposomes are normally cleared rapidly from circulation by the reticuloendothelial system. Liposome half-life in the circulation can be substantially lengthened if gangliosides or lipids derived from polyethylene glycol are inserted in the lipid bilayer. Liposomes coated with polyethylene glycol in this way are not readily recognised by macrophages; they are known as sterically stabilised or stealth liposomes.

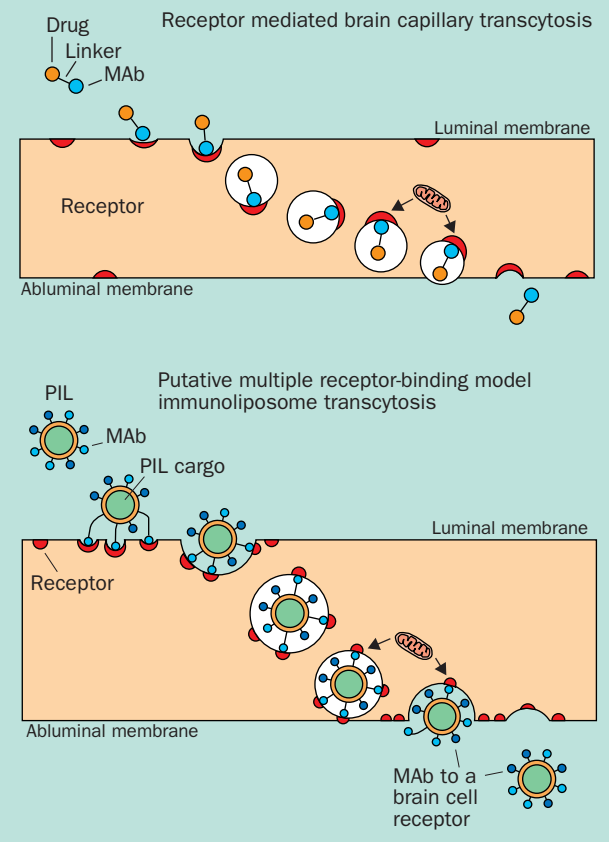

Figure 4. Top: the receptor-mediated transcytosis of a drug of large molecular size by a MAb-linked delivery system. The drug–MAb conjugate binds to a single receptor on the luminal membrane, undergoes endocytosis, and traverses the capillary cytoplasm in a pinocytotic vesicle. Exocytosis to the CNS at the abluminal membrane is thought to be energy dependent. Separation of the drug and MAb by a linker is thought to decrease steric hindrance and promote efficient receptor binding. Bottom: the putative multiple-receptor-binding model of polyethylene-glycol (PEG)-coated-immunoliposome (PIL) transcytosis. Multiple PEG-linked MAbs bind to their receptor targets on the luminal capillary membrane before the PIL undergoes endocytosis. Electron micrographs of lymphocyte transcytosis[62] show that the vesicular membrane may be in contact with the liposome border. Exocytosis at the abluminal membrane is thought to be energy dependent. A second MAb may be attached to some of the PEG strands to target the PIL to a second receptor located on specific cells within the brain.

The exact mechanism by which an immunoliposome of about 80 nm in diameter traverses the 300–400 nm thickness of the brain-capillary endothelial cell[60] is not fully understood. However, unlike receptor-mediated transcytosis, the process presumably begins with the binding of more than one immunoliposome ligand (such as OX26) to multiple capillary luminal membrane receptors (such as the transferrin receptor; figure 4). The coupling of 30 OX26 antibodies per polyethylene-glycol-coated liposome resulted in optimum brain delivery,[61] which suggests that multiple antibody-receptor binding events are needed before transcellular delivery of the formation of liposome to the brain parenchyma is achieved.

We hypothesise that instead of formation of one small isolated pinocytotic vesicle (as in the traditional receptor-mediated transcytosis process), a series of vesicular pits, induced by receptor-ligand binding, are brought into close association (figure 4). This series of local changes in membrane function (around each activated receptor) presumably fuse together forming a single large vesicle, which uses the same transcytosis mechanism to deliver an immunoliposome to the abluminal membrane border. Exocytosis of the polytheylene-glycol-coated liposome at the abluminal membrane is also presumably an ATP-dependent process. Direct engulfment of lymphocytes by brain-capillary endothelial cells in studies with electron microscopy shows that transcytosis of large structures such as leucocytes and liposomes could be possible.[62] Furthermore, if immunoliposomes and lymphocytes use similar receptor-mediated mechanisms to gain access to the brain, treatments designed to downregulate the endothelial receptors used by migrating lymphoid cells could be developed to lower the cellular infiltration seen in disorders such as demyelinating diseases.

Immunoliposome delivery of drugs to the brain

Huwyler and colleagues[61] showed that liposomes of 85 nm diameter could be sterically stabilised with a 2 kDa polyethylene glycol, which contained a lipid at one pole and a maleimide moiety at the pole external to the liposome. OX26 was then coupled by a thioether linkage to the maleimide moiety of these stealth liposomes to form immunoliposomes.[61] OX26 was chosen because it binds to the brain-capillary transferrin receptor, and it had been successfully used as the vector for delivery of other large molecules across the BBB. No brain uptake of polyethylene-glycol-conjugated liposomes (without OX26) containing radioactive daunomycin was observed. However, brain delivery of OX26–polyethylene-glycol-linked immunoliposomes containing radioactive daunomycin was shown. Since a single liposome can carry up to 10 000 drug molecules, the immunoliposome delivery system has the ability to increase drug delivery to the brain by up to four orders of magnitude.[61] Subsequent studies with confocal fluorescence microscopy showed that OX26-conjugated

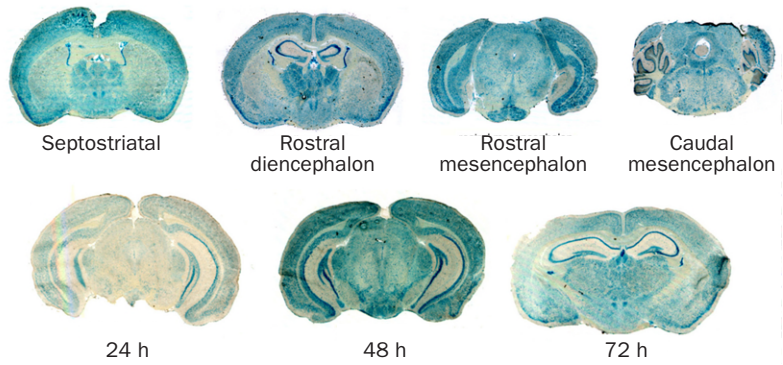

Figure 5. Successful delivery of an exogenous β-galactosidase gene into rat brain by the intravenously injected polyethylene-glycol-coated immunoliposome delivery system. Top: Coronal sections at different levels of the brain show that galactosidase gene expression is detected widely throughout the cortical and subcortical structures at all levels of the brain. Bottom: β-galactosidase histochemistry is shown in brain removed at 24, 48, and 72 h after a single intravenous injection of liposomes carrying β-galactosidase and targeted by 8D3, an MAb to the mouse transferrin receptor. Reproduced with the permission of the National Academy of Sciences USA.[69]

immunoliposomes bind to the luminal and abluminal brain capillary membranes in rats, undergo endocytosis, and release their cargo within rat glioma cells.[63–65]

This delivery system could be important for neuropharmaceutical drug delivery because it permits brain targeting of the liposomally encapsulated drug, and consequently offers a significant reduction in side-effects. Compounds with excellent neuropharmaceutical potential in vitro, which may have been rejected for clinical use because of low brain delivery or some peripheral systemic side-effects, may now be re-evaluated for potential use in conjunction with this delivery vector. Since the liposome capsule undergoes lipolytic degradation to release its contents, the drug is delivered without the use of disulphide or ester linkages, which can significantly affect pharmacological actions.

Immunoliposome delivery of genes to the brain

Inefficient delivery and expression of therapeutic DNA inhibits the potential of gene therapy.[66] Possible methods for delivery of gene therapeutics specifically to the human brain include invasive craniotomy, cationic-liposome–DNA complexes, or viral vectors; but each technique has some limitations.[37] Gene therapy would ideally use a non-viral formulation, intravenously administered, with widespread gene expression throughout the brain, and specific localisation to the brain with no expression in peripheral tissues. In theory, gene expression in the brain could be altered with the targeting techniques discussed above, in combination with gene promoters specific to particular cells.[37]

Because OX26, which is a mouse MAb to the rat transferrin receptor, is not an effective brain delivery vector in mice, MAb to the mouse transferrin receptor have been developed. 8D3, an MAb to the mouse transferrin receptor, immunocytochemically identified microvessels in mouse brain, and had optimum characteristics for drug-targeting studies in mouse models of human disease.[67] β-galactosidase was the test gene selected for vector delivery, because success or failure of the gene delivery system could be readily imaged with histochemical examination of whole brain sections. After a single intravenous injection of immunoliposome-delivered β-galactosidase, the galactosidase was highly expressed throughout the brain (figure 5). Other rat tissues, such as the spleen and liver, also express the transferrin receptor, and β-galactosidase expression was also seen in these sites.[68] Thus, widespread expression of an exogenous gene in brain and peripheral tissues has been induced after a single intravenous administration of plasmid DNA packaged within polyethylene-glycol-coated immunoliposomes.[68]

To produce tissue-specific expression of galactosidase in the brain rather than the spleen, further investigations used 8D3-targeting delivery in conjunction with tissue-specific promoters (figure 5). When the β-galactosidase was delivered with immunoliposomes and simian virus 40 promoter, histochemical analyses showed expression in the brain, spleen, and liver.[68] By contrast, when the same delivery system was used in conjunction with a brain-specific gene promoter for glial fibrillary acidic protein, the β-galactosidase expression was restricted to the brain.[69] With the use of a glial-cell specific promoter, the promise of molecular biological advances has moved into the arena of in vivo application. In gene-therapy trials, safety concerns reinforce the need for newly developed delivery systems, including non-viral vectors such as immunoliposomes.[7] The efficiency of viral vectors for gene therapy across the BBB has yet to be shown. To circumvent the BBB, administration of the vector via intracerebral injection has been tested in recurrent brain tumours.[70] The limited success may be due to poor diffusion of the vector from the injection site, which limits distribution of the therapeutic gene to most cancer cells. In addition, the pre-existing immunity to viral vectors causes inflammation and demyelination in the brain in the apparent absence of lytic infections.[71,72]

Intracranial versus delivery-system-mediated treatment of brain tumours

One approach to the treatment of brain tumours makes use of the fact that certain tumours have larger numbers of transferrin receptors than others. An example of transferrin-mediated delivery without the use of a BBB transit vector comes from studies with a conjugate of transferrin and a point mutant of diphtheria toxin, Tf-CRM107. This toxin selectively kills cells that express high concentrations of the transferrin receptor. When Tf-CRM107 was infused into malignant brain tumours, about half of the patients showed tumour responses. But neurological deficits, consistent with generalised endothelial damage in the brain, were also seen in association with high doses,[73] even though peripheral toxicity was less with intracranial administration. The possibility that chloroquine coadministration could reduce the toxicity of Tf-CRM107[74] is under investigation, but this situation illustrates the type of unexpected problems that may be encountered in attempting

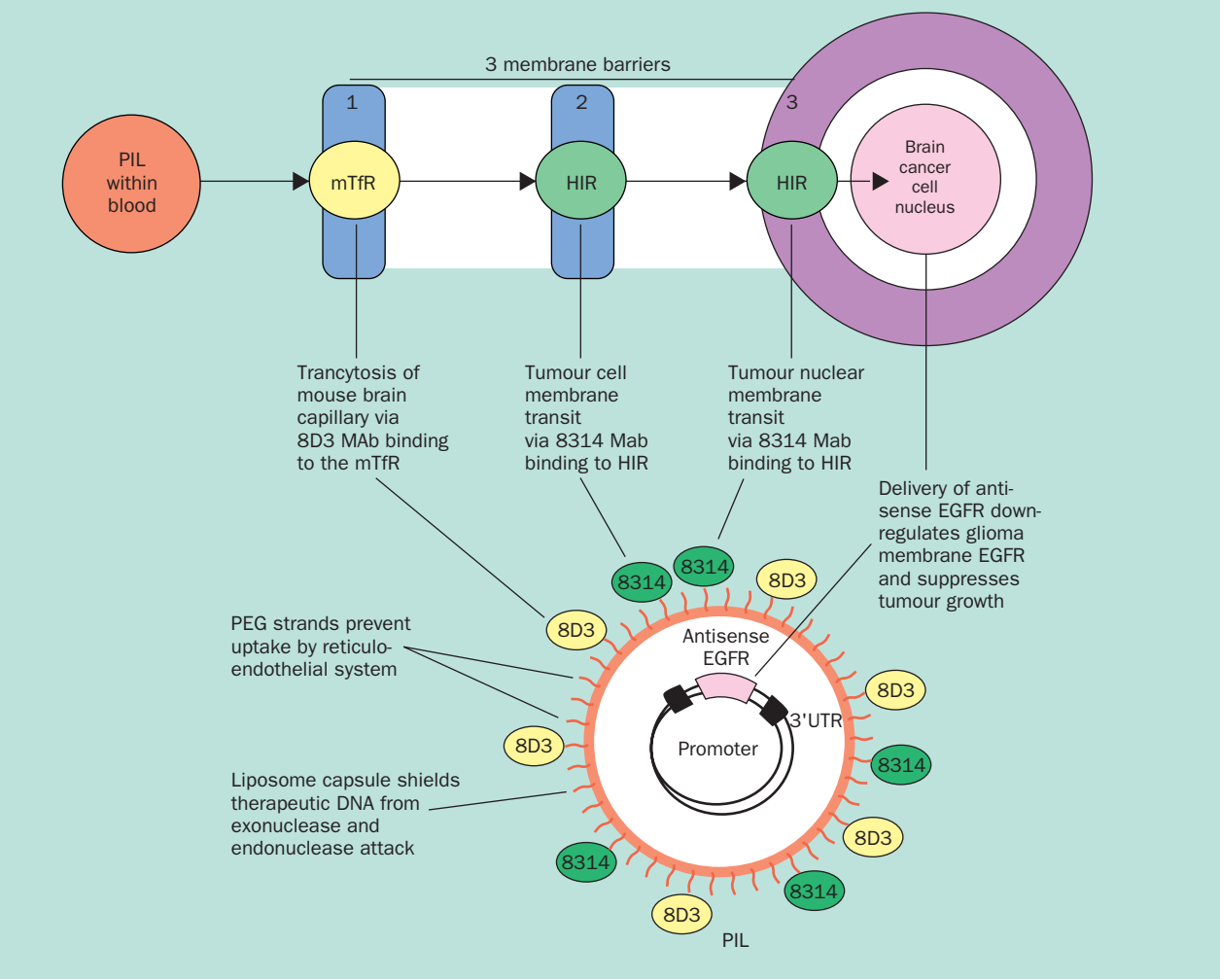

Figure 6. Gene therapy for brain cancer delivered by a polyethylene glycol (PEG) coated immunoliposome (PIL) delivery system. The encapsulation of the therapeutic EGF receptor (EGFR) antisense plasmid DNA within a "nanocontainer," such as an 85 nm PIL, prevents degradation of the therapeutic DNA by the ubiquitous endonucleases present in vivo. MAb is attached to the tip of about 2% of these PEG strands, which also prevent uptake of the PIL in the peripheral capillaries and absorption of serum proteins. Inclusion of the 3′ untranslated region (3′UTR) of the GLUT1 glucose transporter raises expression of the antisense gene by four times. To reach the nuclear compartment of the glioma cell, the intravenously administered PIL must traverse three biological barriers. (1) 8D3 binds to the mouse transferrin receptor (mTfR) to enable the PIL to cross the BBB. (2) The human tumour-cell membrane is traversed by the murine 8314 MAb to the human insulin receptor (HIR), and (3) the MAb to HIR also allows the PIL to cross the the tumour-cell nuclear membrane.[79]

to circumvent the BBB. Nevertheless, intrathecal or intratumoral administration of targeted toxins continues to be actively evaluated. A fusion protein against the interleukin-4 receptor, which contains a modified *Pseudomonas aeruginosa* toxin, has been developed to test this targeted toxin in patients with recurrent malignant astrocytomas.[75]

High-grade astrocytomas overexpress the EGF receptor,[76,77] and antisense gene therapy directed at this receptor is currently being developed. Pardridge and colleagues applied their non-viral targeted gene-delivery system[68,69] to an experimental human brain tumour model (figure 6). The targeted gene-delivery system uses an artificial virus with the EGF-receptor antisense gene inserted into a non-viral expression plasmid, which is encapsulated within a polyethylene-glycol-coated immunoliposome of diameter 85 nm. The surface of the liposome gene-delivery system was supplied with two different peptidomimetic MAbs for transport across the several biological membranes that separate the blood from the nuclear compartment of the cancer cell. 8D3 facilitates transport of the liposome across the murine BBB. The second barrier, the human glioma-cell membrane, is traversed by the murine MAb to the human insulin receptor, 8314.[78] The second MAb also enables the liposome to traverse the third barrier, the tumour-cell nuclear membrane, as the antibody is carried by the normal intracellular trafficking of insulin to the nucleus.[78] In this novel study of therapy for brain tumours, the receptor-targeted therapeutic EGF-receptor antisense nucleic acid (the magic bullet of 21st-century molecular neurobiology) was intravenously delivered with an artificial virus[78] (the magic gun)[37] to the nuclei of glioma cells (figure 6).

Mice that had been implanted with intracranial U87 human gliomas were treated with this expression plasmid encoding antisense mRNA against the EGF receptor. Mice

treated intravenously once a week (for 4 weeks) with the EGF-receptor antisense gene therapy lived twice as long as control mice. Controls were treated with either a luciferase gene or saline. The presence of brain tumours in surviving mice (which had received gene therapy) was confirmed at autopsy, and massive tumour growth was confirmed in the control mice.[79] The success of this animal model study raises hope that the human counterpart will be forthcoming. In tumours in which high expression of the EGF receptor apparently confers resistance to radiotherapy, the antisense treatment could be augmented by radiation treatments. Furthermore, brain cancer genomics programs have identified other genes, such as interleukins 4 and 13,[75,80] that are overexpressed in these primary brain tumours. These findings show that a second antisense treatment directed at another gene target might also amplify the therapeutic effect in brain tumours.

Differences in vector targeting in different species

Although the animal models described above have shown the potential of the methods, human therapeutic systems have yet to be sufficiently developed for clinical trials. BBB antibody targeting vectors are species specific. In mice, 8D3 is the chimeric peptide of choice, with specificity for the mouse transferrin receptor. In rats, it is OX26, and in rhesus monkeys, the 8314 MAb, which also recognises the (human) brain capillary insulin receptor. Adaptation of this technology to human beings would be greatly accelerated by development of a genetically engineered version of the MAb to the human insulin receptor.[53] The promising treatment modalities discussed above are strongly supported by neuroimaging studies and investigations in animal models of stroke and brain tumours,[2,8] but only clinical trials can truly evaluate their usefulness in human beings.

Conclusions

The search for new methods of achieving BBB transit of specific brain-targeted compounds is just beginning, but the remarkable progress of the past decade suggests that the ability to deliver BBB-impermeable agents across the brain endothelium is an attainable goal. Vector-mediated transport and the CNS-directed delivery of immunoliposomes are two techniques of great promise. The challenge for the next decade is to meld these BBB drug-delivery techniques to the development of traditional pharmaceuticals for the purpose of testing and generating a variety of new neuro-pharmaceuticals.[81]

The BBB delivery of antisense-based therapeutics is an area of great potential, and it is likely to be a new focus of intense interest in the coming decade. The genetic loci for many inherited neurological diseases have been defined over the past few years, and there will now be a search for restorative treatments, including gene-delivery strategies.

A second generation of BBB-vector-mediated transport investigations seems to be on the horizon, inspired by the feasibility of receptor-based treatments in animal models of neurological disease. Receptor-based therapies have to allow transit of newly acquired therapeutics not only across the BBB but also across the neuronal and glial membranes, which may become a second focus for brain-directed drug delivery; the linking of capillary and neuropil membrane-based transit vectors will be the next challenge. In gene-delivery studies, the use of a glial-cell-specific promoter region has already been successful in a mouse model. Neuronal-specific promoter regions may be applied to other neurological diseases in the near future. Tissue-specific gene promoters (unique to myelinated or cholinergic and adrenergic cells, for example) have the potential to restrict expression to a desired site. These studies collectively suggest that the promise of brain-targeting strategies, and of molecular biology, may very soon be adapted to clinical neurology. Advances in brain-targeting methods could spearhead the progress of both academic neuroscience and the neuropharmaceutical industry in the next two decades.[81] Indeed the potential success of large molecule receptor-based neurotherapeutics seems to be greater when they are developed in conjunction with brain delivery methods; this suggests that we can continue to anticipate rapid advances in our understanding of brain capillary biology.

Search strategy and selection criteria

Data for this review were identified by searches of Medline with the terms "brain delivery", "immunoliposome", "transferrin receptor", "insulin receptor", "transcytosis", and "brain capillary"; references from relevant articles; and searches from the files of the authors.Only papers published in English were reviewed.

Acknowledgments

We thank William Pardridge for providing figures 2, 3, and 5, and for many helpful discussions. We also thank Dan Cornford for preparation of figures 1, 4, and 6. This paper is revised and updated from a presentation given by EMC to the second WTA Daejon Technomart International Symposium, Daejon, South Korea, Nov 8, 2001.

Author contributions

Both authors contributed equally to this work.

Conflict of interest

We have no conflict of interest.

Role of the funding source

Support from National Institute of Health grant NS 37360 and the Veterans Administration to EMC during the preparation of this paper is gratefully acknowledged.

References

1 Cornford EM, Hyman S. Blood-brain barrier permeability to small and large molecules. *Adv Drug Del Rev* 1999; **36**: 145–63.
2 Pardridge WM. Drug and gene targeting to the brain with Trojan horses. *Nature Rev* 2000; **1**: 131–39.
3 Stewart PA, Hayakawa K, Farrell CL. Quantitation of blood-brain barrier ultrastructure. *Microsc Res Tech* 1994; **27**: 516–27.
4 Minn A, Ghersi-Egea JF, Perrin R, Leninger B, Siest G. Drug metabolizing enzymes in the brain and cerebral microvessels. *Brain Res Rev* 1991; **16**: 65–82.
5 Abbott NJ, Romero IA. Transporting therapeutics across the blood-brain barrier. *Mol Med Today* 1996; **2**: 106–13.
6 Pardridge WM, Golden PL, Kang YS, Bickel U. Brain microvascular and astrocyte localization of P-glycoprotein. *J Neurochem* 1997; **68**: 1278–85.
7 Rubanyi GM. The future of human gene therapy. *Mol Aspects Med* 2001; **22**: 113–42.
8 Pardridge WM. Targeting neurotherapeutic agents through the blood-brain barrier. *Arch Neurol* 2002; **59**: 35–40.
9 Li JY, Boado RJ, Pardridge WM. Blood-brain barrier genomics. *J Cereb Blood Flow Metabol* 2001; **21**: 61–68.
10 Shusta EV, Boado RJ, Mathern GW, Pardridge WM. Vascular genomics of the human brain. *J Cereb Blood Flow Metabol* 2002; **22**: 245–52.
11 Lee CG, Gottsman MM, Cardarelli CD, et al. HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* 1998; **37**: 3594–601.
12 Takasawa K, Terasaki T, Suzuki H, Sugiyama Y. In vivo evidence for carrier mediated efflux transport of 3'-azido-3'-deoxythymidine and 2',3'-dideoxyinosine

across the blood-brain barrier via a probenecid-sensitive transport system. *J Pharmacol Exp Ther* 1997; **281**: 369–75.
13 Wu D, Clement JG, Pardridge WM. Low blood-brain barrier permeability to azidothymidine (AZT), 3TC, and thymidine in the rat. *Brain Res* 1998; **791**: 313–16.
14 Pardridge WM. Peptide drug delivery to the brain. New York: Raven Press, 1991: 357.
15 Stewart PA. Endothelial vesicles in the blood-brain barrier: are they related to permeability? *Cell Mol Neurobiol* 2000; **20:** 149–63.
16 Predescu D, Palade GE. Plasmalemmal vesicles represent the large pore system of continuous microvascular endothelium. *Am J Physiol* 1993; **265**: H725–33.
17 Ghitescu L, Bendayan M. Transendothelial transport of serum albumin: a quantitative immunocytochemical study. *J Cell Biol* 1992; **117**: 745–55.
18 Schnitzer JE, Oh P, Pinney E, Allard J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol* 1994; **127**: 1217–32.
19 Sheff DR, Daro EA, Hull M, Mellman I. The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. *J Cell Biol* 1999; **145**: 123–29.
20 Ningaraj NS, Rao M, Hashizume K, Asotra K, Black KL. Regulation of blood-brain tumour barrier permeability by calcium-activated potassium channels. *J Pharmacol Exp Ther* 2002; **301**: 1–4.
21 Pardridge WM, Kumagai AK, Eisenberg JB. Chimeric peptides as a vehicle for peptide pharmaceutical delivery through the blood-brain barrier. *Biochem Biophys Res Commun* 1987; **146**: 307–15.
22 Kumagai AK, Eisenberg JB, Pardridge WM. Absorptive mediated endocytosis of cationized albumin and a β-endorphin-cationized albumin chimeric peptide by isolated brain capillaries. *J Biol Chem* 1987; **262:** 15214–19.
23 Pardridge WM, Triguero D, Buciak JL, Yang J. Evaluation of cationized rat albumin as a potential blood-brain barrier transport vector. *J Pharmacol Exp Ther* 1990; **255**: 893–99.
24 Pardridge WM, Triguero D, Buciak JL. β-Endorphin chimeric peptides; transport through the blood-brain barrier in vivo and cleavage of disulfide linkage by brain. *Endocrinology* 1990; **126:** 977–984.
25 Kang YS, Pardridge WM. Brain delivery of biotin bound to a conjugate of neutral avidin and cationized albumin. *Pharm Res* 1994; **11**: 1257–64.
26 Broadwell RD, Baker-Cairns BJ, Friden PM, Oliver C, Villegas JC. Transcytosis of protein through the mammalian cerebral epithelium and endothelium: III, receptor-mediated transcytosis through the blood-brain barrier of blood-borne transferrin and antibody against the transferrin receptor. *Exp Neurol* 1999; **142**: 47–55.
27 Fukuta M, Okada H, Inuma S, Yanai H, Toguchi H. Insulin fragments as carrier for peptide delivery across the blood brain barrier. *Pharm Res* 1994; **11**: 1681–88.
28 Shin SU, Friden P, Moran M, Olson T, Kang YS, Pardridge WM, Morrison SL. Transferrin-antibody fusion proteins are effective in brain targeting. *Proc Natl Acad Sci USA* 1995; **92**: 2820–24.
29 Park E, Starzyk RM, McGrath JP, et al. Production and characterization of fusion proteins containing transferrin and nerve growth factor. *J Drug Target* 1998; **6**: 53–64.
30 Singh M. Transferrin as a targeting ligand for liposomes and anticancer drugs. *Curr Pharm Design* 1999; **5**: 443–51.
31 Ali SA, Joao HC, Mammerschmid F, Eder J, Steinkasserer A. Transferrin trojan horses as a rational approach for the biological delivery of therapeutic peptide domains. *J Biol Chem* 1999; **274**: 24066–73.
32 Li H, Qian ZM. Transferrin/transferrin receptor-mediated drug delivery. *Med Res Rev* 2002; **22**: 225–50.
33 Friden PM, Walus L, Musso GF, Taylor MA, Malfroy B, Starzyk RM. Antitransferrin receptor antibody and antibody-drug conjugates cross the blood brain barrier. *Proc Natl Acad Sci USA* 1991; **88**: 4771–75.
34 Bickel U, Yoshikawa T, Landaw EM, Faull KF, Pardridge WM. Pharmacologic effects in vivo in brain by vector-mediated drug delivery. *Proc Natl Acad Sci USA* 1993; **90**: 2618–22.
35 Wu D, Pardridge WM. Central nervous system pharmacologic effect in conscious rats after intravenous injection of a biotinylated vasoactive intestinal peptide analog coupled to a blood-brain barrier drug delivery system. *J Pharmacol Exp Ther* 1996; **279**: 77–83.
36 Huwyler J, Pardridge WM. Examination of blood-brain barrier transferrin receptor by confocal fluorescent microscopy of unfixed isolated rat brain capillaries. *J Neurochem* 1998; **70:** 883–86.
37 Pardridge WM. Brain drug targeting. New York: Cambridge University Press, 2001: 353.
38 Li XB, Liao GS, Shu YY, Tang XS. Brain delivery of biotinylated NGF bounded to an avidin-transferrin conjugate. *J Nat Toxins* 2000; **9:** 73–83.
39 Pardridge WM, Kang YS, Buciak JL. Transport of human recombinant brain-derived neurotrophic factor (BDNF) through the rat blood-brain barrier in vivo using vector-mediated peptide drug delivery. *Pharm Res* 1994; **11:** 738–46.
40 Granholm AC, Backman C, Bloom F, et al. NGF and anti-transferrin receptor antibody conjugate: short and long term effects on survival of cholinergic neurons in intraocular septal transplants. *J Pharmacol Exp Ther* 1994; **268:** 448–59.
41 Wu D, Boado R, Pardridge WM. Pharmacokinetics of blood-brain barrier transport of [^{3}H]-biotinylated phosphorothioate oligodeoxynucleotide conjugated to a vector-mediated drug delivery system. *J Pharmacol Exp Ther* 1996; **276:** 206–11.
42 Pardridge WM, Boado R, Kang YS. Vector mediated delivery of a peptide nucleic acid through the blood-brain barrier in vivo. *Proc Natl Acad Sci USA* 1995; **92:** 5592–96.
43 Deguchi Y, Kurihara A, Pardridge WM. Retention of biologic activity of human epidermal growth factor following conjugation to a blood-brain barrier drug delivery vector via an extended poly (ethylene glycol) linker. *Bioconjug Chem* 1999; **10:** 32–37.
44 Kang YS, Voight K, Bickel U. Stability of the disulfide bond in an avidin-botin linked chimeric peptide during in vivo transcytosis through brain endothelial cells. *J Drug Target* 2000; **8:** 425–34.
45 Zhang Y, Pardridge WM. Mediated efflux of IgG molecules from brain to blood across the blood-brain barrier. *J Neuroimmunol* 2001; **114:** 168–72.
46 Wu D, Pardridge WM. Neuroprotection with noninvasive neurotrophin delivery to brain. *Proc Natl Acad Sci USA* 1999; **96**: 254–59.
47 Zhang Y, Pardridge WM. Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system. *Stroke* 2001; **32:** 1378–83.
48 Keep RF. Editorial comment. *Stroke* 2001; **32:** 1383–84.
49 Zhang Y, Pardridge WM. Conjugation of brain derived neurotrophic factor to a blood-brain barrier drug targeting system enables neuroprotection in regional brain ischemia following intravenous injection of the neurotrophin. *Brain Res* 2001; **889:** 49–56.
50 Song BW, Vinters HV, Wu D, Pardridge WM. Enhanced neuroprotective effects of basic fibroblast growth factor in regional brain ischemia after conjugation to a blood-brain barrier delivery vector. *J Pharmacol Exp Ther* 2002; **301**: 605–10.
51 Boado RJ, Tsukamoto H, Pardridge WM. Drug delivery of antisense molecules to the brain for treatment of Alzheimer's disease and cerebral AIDS. *J Pharm Sci* 1998; **87:** 1308–15.
52 Shi N, Boado RJ, Pardridge WM. Antisense imaging of gene expression in the brain in vivo. *Proc Natl Acad Sci USA* 2000; **97:** 14709–14.
53 Coloma MJ, Lee HJ, Kurihara A, et al. Transport across the primate blood-brain barrier of a genetically engineered chimeric monoclonal antibody to the human insulin receptor. *Pharm Res* 2000; **17:** 266–74.
54 Kurihara A, Pardridge WM. Imaging brain tumours by targeting peptide radiopharmaceuticals through the blood-brain barrier. *Cancer Res* 1999; **59:** 6159–63.
55 Kurihara A, Deguchi Y, Pardridge WM. Epidermal growth factor radiopharmaceuticals: 111In chelation, conjugation to a blood-brain barrier delivery vector via a biotin-polyethylene linker, pharmacokinetics, and in vivo imaging of experimental brain tumours. *Bioconjug Chem* 1999; **10**: 502–11.
56 Lee HJ, Zhang Y, Zhu C, Duff K, Pardridge WM. Imaging brain amyloid of Alzheimer's disease in vivo in transgenic mice with an Ab peptide radiopharmaceutical. *J Cereb Blood Flow Metabol* 2002; **22**: 223–31.
57 Huang A, Kennel SJ, Huang L. Immunoliposome labeling: a sensitive and specific method for cell surface labeling. *J Immunol Methods* 1981; **46**: 141–51.
58 Huang A, Kennel SJ, Huang L. Interactions of immunoliposomes with target cells. *J Biol Chem* 1983; **258**: 14034–40.
59 Ho RJ, Huang L. Interactions of antigen-sensitized immunoliposomes with immobilized antibody: a homogeneous solid phase immunoliposome assay. *J Immunol* 1985; **134:** 4035–40.
60 Cornford EM, Hyman S, Cornford ME, Landaw EM, Delgado-Escueta AV. Interictal seizure resections show two configurations of endothelial Glut1 glucose transporter in the human blood-brain barrier. *J Cereb Blood Flow Metabol* 1998; **18:** 26–42.
61 Huwyler J, Wu D, Pardridge WM. Brain drug delivery of small molecules using immunoliposomes. *Proc Natl Acad Sci USA* 1996; **93**: 14164–69.
62 Faustmann PM, Dermeitzel R. Extravasation of polymorphonuclear leukocytes from the cerebral microvasculature. *Cell Tissue Res* 1985; **242**: 399–407.
63 Huwyler J, Yang J, Pardridge WM. Targeted delivery of daunomycin using immunoliposomes: pharmacokinetics and tissue distribution in the rat. *J Pharmacol Exp Ther* 1997; **282:** 1541–46.
64 Huwyler J, Pardridge WM. Examination of the blood-brain barrier transferrin receptor by confocal fluorescence microscopy of unfixed isolated brain capillaries. *J Neurochem* 1998; **70**: 883–86.
65 Cerletti A, Drewe J, Fricker G, Eberle AN, Huwyler J. Endocytosis of an immunoliposome-based drug delivery system. *J Drug Target* 2000; **8:** 435–36.
66 Anderson WF. Human gene therapy. *Nature* 1998; **392**: 25–30.
67 Lee HJ, Engelhardt B, Lesley J, Bickel U, Pardridge WM. Targeting rat anti-mouse transferrin receptor monoclonal antibodies through blood-brain barrier in mouse. *J Pharmacol Exp Ther* 2000; **292**: 1048–52.
68 Shi N, Boado RJ, Pardridge WM. Receptor-mediated gene targeting to tissues in vivo following intravenous administration of pegylated immunoliposomes. *Pharm Res* 2001; **18:** 1091–95.
69 Shi N, Zhang Y, Zhu C, Boado RJ, Pardridge WM. Brain-specific expression of an exogenous gene after intravenous administration. *Proc Natl Acad Sci USA* 2001; **98:** 12754–59.
70 Ram Z, Culver KW, Oshiro EM, et al. Therapy of malignant brain tumours by intratumour implantation of retroviral vector-producing cells. *Nat Med* 1997; **3**: 1354–61.
71 Dewey RA, Morrisey G, Cowsill CM, et al. Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase in survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: implications for clinical trials. *Nat Med* 1999; **5:** 1256–63.
72 Driesse MJ, Vincent AJ, Sillevus Smitt PA, et al. Intracerebral injection of adenovirus harboring the HSVtk gene combined with gangciclovir administration: toxicity study in non-human primates. *Gene Ther* 1998; **5**: 1122–29.
73 Laske DW, Youle RJ, Oldfield EH. Tumour regression with regional distribution of the targeted toxin Tf-CRM107 in patients with malignant brain tumours. *Nat Med* 1997; **3**: 1362–68.
74 Hagihara N, Wallbridge S, Olson AW, Oldfield EH, Youle RJ. Vascular protection by chloroquine during brain tumour therapy with Tf-CRM107. *Cancer Res.* 2000; **60:** 230–34.
75 Hall WA. Targeted toxin therapy for malignant astrocytomas. *Neurosurgery* 2000; **46**: 544–51.
76 Libermann TA, Nusbaum HR, Razon N, et al. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* 1985; **313:** 144–47.
77 Wong AJ, Bigner SH, Bigner DD, Kinzler KW, Hamilton SR, Vogelstein B. Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. *Proc Natl Acad Sci USA* 1987; **84**: 6899–903.
78 Zhang Y, Lee HJ, Boado RJ, Pardridge WM. Receptor-mediated delivery of an antisense gene to human brain cancer cells. *J Gene Med* 2002; **4**: 183–94.
79 Zhang Y, Zhu C, Pardridge WM. Antisense gene therapy of brain cancer with an artificial virus gene delivery system. *Mol Ther* 2002; **6:** 67–72.
80 Debinski W, Gibo DM, Slagle B, Powers SK, Gillespie GY. Receptor for interleukin 13 is abundantly and specifically over-expressed in patients with glioblastoma multiforme. *Int J Oncol* 1999; **15**: 481–86.
81 Lawrence RN. William Pardridge discusses the lack of BBB research. *Drug Dis Today* 2002; **7**: 223–26.