

Targeted Drug Delivery via the Transferrin Receptor-Mediated Endocytosis Pathway

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Abstract—The membrane transferrin receptor-mediated endocytosis or internalization of the complex of transferrin bound iron and the transferrin receptor is the major route of cellular iron uptake. This efficient cellular uptake pathway has been exploited for the site-specific delivery not only of anticancer drugs and proteins, but also of therapeutic genes into proliferating malignant cells that overexpress the transferrin receptors. This is achieved either chemically by conjugation of transferrin with therapeutic

drugs, proteins, or genetically by infusion of therapeutic peptides or proteins into the structure of transferrin. The resulting conjugates significantly improve the cytotoxicity and selectivity of the drugs. The coupling of DNA to transferrin via a polycation or liposome serves as a potential alternative to viral vector for gene therapy. Moreover, the OX26 monoclonal antibody against the rat transferrin receptor offers great promise in the delivery of therapeutic agents across the blood-brain barrier to the brain.

I. Introduction

The rapid development in current pharmaceutical drug discovery has resulted in the emergence of increasing numbers of novel therapeutic drugs for the treatment of a variety of diseases. However, at present the main problem associated with systemic drug administration is likely to include even biodistribution of pharmaceuticals throughout the body, the lack of drug-specific affinity toward a pathological site, nonspecific toxicity, and other side effects resulting from high doses. An attractive strategy to enhance the therapeutic index of drugs is to specifically deliver these agents to the defined target cells thus keeping them away from healthy cells, which are sensitive to the toxic effects of the drugs. Polymer- and liposome-based delivery systems show potentials as specific and target-oriented delivery systems (Langer, 1998; Maruyama et al., 1999; Vyas and Sihorkar, 2000; Vyas et al., 2001). Naturally existing proteins (such as transferrin) have also received major attention in the area of drug targeting since these proteins are biodegradable, nontoxic, and nonimmunogenic. Moreover, they can achieve site-specific targeting due to the high amounts of their receptors present on the cell surface. The efficient cellular uptake of transferrin (Tf¹) pathway has shown potential in the delivery of anticancer drugs, proteins, and therapeutic genes into primarily proliferating malignant cells that overexpress transferrin receptors (TfRs) (Kratz and Beyer, 1998b; Singh, 1999; Vyas and Sihorkar, 2000; Kircheis et al., 2002).

¹ Abbreviations: Tf, transferrin; TfR, Tf receptor; oTf, ovotransferrin; Lf, lactoferrin; p97, melanotransferrin; IRP, iron-regulatory protein; IRE, iron-responsive element; HFE, hemochromatosis protein (the protein mutated in hereditary hemochromatosis); β_2 M, β_2 -microglobulin; kb, kilobase(s); SFT, stimulator of iron transport; BBB, blood-brain barrier; DMT1, divalent metal transporter 1; GPI, glycosylphosphatidylinositol; MMC, mitomycin C; PEG, polyethylene glycol; α -IFN, α -interferon; PLL, polylysine; ILP, immunoliposome; pHPMA, poly-[N-(2-hydroxypropyl)methacrylamide]; GFP, green fluorescent protein; HSV-TK, herpes simplex virus thymidine kinase; SA, streptavidin; MBS, *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester; SPDP, *N*-succinimidyl-3-[2-pyridyldithio]propionate; BDNF, brain-derived neurotrophic factor; VIPa, vasoactive intestinal peptide analog; mAb, monoclonal antibody; MCAO, middle cerebral artery occlusion; PNA, polyamide nucleic acid; ODN, antisense oligonucleotides; PO, phosphodiester; PS, phosphorothioate.

In this review, we summarize the biochemistry and molecular biology of Tf and the TfR, including the structure, function, and regulation of TfR expression as well as the latest progress in understanding the mechanism of TfR-mediated iron uptake by cells and transport across the blood-brain barrier. In particular, we address the role of Tf and TfR in the targeted delivery of therapeutic drugs, including small molecule drugs, therapeutic peptides, proteins, and even genes, into the malignant tissues or cells. The role of TfR in brain drug targeting and delivery is also included.

II. Transferrins

During the past decades, intensive studies have been made toward understanding these unique iron-binding proteins. Several reviews have given a broad range of coverage of Tf and TfR functional properties, structures, metal binding properties, and their potential in biomedical processes (Baker, 1994; Morgan, 1996; Richardson and Ponka, 1997; Aisen, 1998; Sun et al., 1999; Andrews, 2000; Lieu et al., 2001).

A. Occurrence and Biological Function

The transferrins comprise a family of large (molecular mass ca. 80 kDa) nonheme iron-binding glycoproteins, believed to originate with the evolutionary emergence of vertebrates or prevertebrates. Three major types of transferrins have been characterized. Serum Tf occurs in blood and other mammalian fluids including bile, amniotic fluid, cerebrospinal fluid, lymph, colostrum, and milk. Ovotransferrin (oTf) is found in avian and reptilian oviduct secretions and in avian egg white (Jeltsch and Chambon, 1982; Williams et al., 1982), and lactoferrin (Lf) is found in milk, tear, saliva, and other secretions (Baggiolini et al., 1970; Metzboutigue et al., 1984). Transferrin is mainly synthesized by hepatocytes, with a concentration of 2.5 mg/ml and 30% occupied with iron in blood plasma (Leibman and Aisen, 1979). Recently, a new member of the transferrin family, melanotransferrin (also called p97), has been identified as an integral membrane protein in human malignant melanoma cells and in some fetal tissues (Brown et al., 1982; Rose et al., 1986). It can also be expressed by a wide range of cultured cell types, including liver and intestinal cells (Qian and Wang, 1998).

The principal biological function of transferrins, except melanotransferrin, is thought to be related to iron binding properties. Serum Tf has the role of carrying iron from the sites of intake into the systemic circulation to the cells and tissues (Morgan, 1964; Baker and Morgan, 1969). It is also likely to be involved in the transportation of wide range of other metal ions other than iron, including therapeutic metal ions, radio diagnostic metal ions, and some toxic metal ions (Savigni and Morgan, 1998; Sun et al., 1999). Lactoferrin, on the other hand, is believed to act principally as a bacteriostat, by chelating the iron, which is essential for the growth of microorganism antimicrobial activity. Lactoferrin may also be involved in modulation of the immune and inflammatory responses and act as a growth factor, which is unrelated to iron binding properties (Iyer and Lönnerdal, 1993; Lönnerdal and Iyer, 1995). Ovotransferrin also exhibits antimicrobial activity. The function of melanotransferrin is not yet clear. It seems true that it plays a small role in iron uptake. Instead, it may help in the rapid proliferation of tumor cells and also act as an iron scavenger at the cell surface to prevent lipid peroxidation (Kwok and Richardson, 2002).

B. General Structural Features

The primary structures of over 10 transferrins have been determined and can be found in various protein databases. Transferrins are single-chain glycoproteins containing ca. 700 amino acids with molecular mass ca. 80 kDa. The sequence identity between different species and different members of the family is extremely high, e.g., 78% identity between rabbit and human serum transferrin, 60% between serum transferrin and lacoferrin, and ca. 40% identity between melanotransferrin and other transferrins. The high levels of conservation in their primary structures were also reflected in their three-dimensional structures. Many crystal structures of transferrins (different species and some fragments) are available and have been reviewed previously (Sun et al., 1999). Briefly, the polypeptide chain is folded into two structurally similar but functionally different lobes, referred to as N- and C-lobe, respectively. Two lobes were connected by a short peptide. Each lobe can be further divided into two domains enclosing a deep hydrophilic cleft bearing an iron binding site. At the metal binding site, Fe^{3+} coordinates with distorted octahedral geometry to two oxygens from Tyr, one nitrogen from His, one oxygen from Asp, and two oxygens from a bidentate carbonate (synergistic anion). The ligands are from two domains and two polypeptide strands, which cross over between the two domains at the back of the iron site. This kind of arrangement is crucial to ensure that the domains are able to move apart to form an open conformation, hinged by the backbone strands, which leads to iron release. Bicarbonate is essential for strong binding of iron to the specific site of Tf and may also have a role in iron release. In addition to iron, many

metal ions other than iron have been found to bind to the specific iron sites (Sun et al., 1999; Zhong et al., 2002), thus Tf has been implicated in the transportation of other metal ions.

Another distinctive feature of Tf is that it undergoes conformational changes during Fe^{3+} uptake and release that are thought to be crucial for the selective recognition by the receptor of the transporter protein. The mechanism for opening and closing the lobes has been studied intensively but still remains elusive. It has been postulated that the dilysine (Lys209–Lys301 for ovotransferrin) pair may serve a special function in the release of iron. The charge repulsion resulting from the protonation of the dilysine, located in opposite domains, at lower pH may be the trigger to open the cleft and facilitate iron release (Dewan et al., 1993). In addition, this dilysine pair may also serve as an anion binding site for iron release (Baker, 1994; He et al., 1999a). Mutation of either or both lysines to glutamate or glutamine would abolish this trigger and result in an extremely slow release of iron compared with that from the intact N-lobe Tf (He et al., 1999a). For human serum Tf N-lobe, the presence of a trigger mechanism for the domain closure has been claimed on the basis of the absence of full closure in the Fe^{3+} -loaded Asp63Ser mutant, as analyzed by X-ray solution scattering (Grossmann et al., 1993a). However, the X-ray crystal structure of Asp60Ser lactoferrin showed a greater domain closure than the parent half-molecule (N-lobe) lactoferrin, which has led to the proposal of an equilibrium between the open and closed forms in solution with a low energy barrier (Faber et al., 1996). Recently, a different trigger mechanism for domain closure was proposed, such that the small triggered motion in Tyr92, an Fe^{3+} -coordinating ligand, would induce the extensive rearrangements in the hydrogen bonding networks in the β -strand where Tyr92 is located. These networks would work as a driving force for the domain closure (Mizutni et al., 2001).

III. The Transferrin Receptors

A. Structure of the Transferrin Receptor 1

The primary structure of the human transferrin receptor 1 (TfR1) has been deduced from the nucleotide sequence of its cDNA (McClelland et al., 1984; Schneider et al., 1984). It is a transmembrane homodimer that consists of two identical monomers with a molecular mass of approximately 90 kDa; each monomer is joined by two disulfide bonds at Cys89 and Cys98 (Jing and Trowbridge, 1987). It has a short, NH_2 -terminal cytoplasmic region (residues 1 to 67), a single transmembrane pass (residues 68 to 88), and a large extracellular portion (ectodomain, residues 89 to 760), which is soluble and bears a trypsin-sensitive site and contains a binding site for transferrin. TfR1 is synthesized in the endoplasmic reticulum and is post-translationally modified with both phosphate and fatty acyl groups (Omary

and Trowbridge, 1981; Schneider et al., 1982). The extracellular domain contains three N-linked glycosylation sites at Asn251, Asn317, and Asn727 and one O-linked glycosylation site at Thr104 (Omary and Trowbridge, 1981). These sites are thought to be crucial for the function of TfR1. Mutations at the N-linked glycosylation sites impair transferrin binding activity. Similarly, elimination of the O-linked glycosylation at Thr104 enhances the cleavage of TfR1 and promotes the release of its ectodomain (Williams and Enns, 1991; Rutledge and Enns, 1996).

Crystallographic studies of the ectodomain of human TfR1 (residues 122 to 760) revealed that homodimer of TfR1 is organized as a butterfly-like shape (Fig. 1). Each TfR1 monomer consists of three distinct globular domains (Lawrence et al., 1999). Three domains, identified as the protease-like, apical, and helical domains, form a lateral cleft, which is likely to be in contact with the docked transferrin molecules (Lawrence et al., 1999). The ectodomain of TfR1 is separated from the membrane by a stalk, which probably includes residues involved in disulfide bond formation and the O-linked glycosylation (Fuchs et al., 1998). The amino acid sequence of the globular ectodomain of TfR1 is 28% identical to that of membrane glutamate carboxypeptidase II, which hydrolyzes the most prevalent mammalian neuropeptide, *N*-acetyl- α -L-aspartyl-L-glutamate (Lawrence et al., 1999). Therefore, it has been suggested that TfR1 is evolved from a peptidase related to membrane glutamate carboxypeptidase II (Bzdega et al., 1997) although it lacks peptidase activity.

A homodimer of TfR1 can bind up to two molecules of Tf. The binding affinity of Tf for various receptors is very

high, from 10^5 to 10^{10} M $^{-1}$ (Sun et al., 1999). Small differences between transferrin receptors can have large effects on the binding affinity for Tf from different mammals and different cells. However, the diferric protein always has a higher affinity for the TfR1 than its monoferric and apo-forms. It is not fully understood how and where TfR binds Tf. The primary receptor recognition site of human transferrin is thought to be mainly on the C-lobe of Tf (Zak et al., 1994). However, recent studies show that both C- and N-lobe of human serum Tf are necessary for receptor recognition (Mason et al., 1997; Zak et al., 2002). Other studies using a human/chicken chimeric TfR1 suggest that Tf binds to a region corresponding to the helical domain (Buchegger et al., 1996). Site-directed mutagenesis has shown that TfR1 residues 646–648, which are present in helix 3 of the helical domain, are critical for Tf binding (Dubljevic et al., 1999). However, docking of Tf molecules to the crystal structure of TfR1 shows that the most contact between Tf and TfR1 involves the apical domain of TfR1 and the N₁ and C₁ domains of Tf, although certain parts of the protease-like and helical domains may also participate in binding of Tf (Lawrence et al., 1999). Therefore, further studies are warranted to understand how TfR1 binds Tf.

B. Regulation of Transferrin Receptor 1 Expression

The TfR1 appears to be expressed in all nucleated cells in the body, such as red blood cells, erythroid cells, hepatocytes, intestinal cells, monocytes (macrophages), brain, the blood-brain barrier (also blood-testis and blood-placenta barriers), and also in some insects and certain bacteria (Levay and Viljoen, 1995; Lönnnerdal and Iyer, 1995; Schryvers et al., 1998; Qian et al., 1998, 1999a, 2000; Moos and Morgan, 2000), but differs in levels of expression (Davies et al., 1981; Enns et al., 1982). It is expressed on rapidly dividing cells, with 10,000 to 100,000 molecules per cell commonly found on tumor cells or cell lines in culture (Inoue et al., 1993). In contrast, in nonproliferating cells, expression of TfR1 is low or frequently undetectable.

Expression of TfR1 in nonerythroid cells is regulated at the post-transcriptional level by interactions of iron-regulatory proteins (IRPs) and iron-responsive elements (IREs) in the 3'-untranslated region of TfR1 mRNA. The 3'-untranslated region of receptor mRNA contains a series of five hairpin stem-loop structures required for iron-dependent regulation (Casey et al., 1988). The stem-loop structures called IREs are recognized by *trans*-acting proteins, known as IRPs (Leibold and Munro, 1988), which control the rate of mRNA translation or stability (Rao et al., 1986; Müllner and Kühn, 1988). Two closely related IRPs (IRP1 and IRP2) have been identified to date (Leibold and Munro, 1988; Henderson et al., 1993). Both display IRE binding properties under conditions of iron deprivation. IRP1 shares 30% sequence identity with *m*-aconitase, a [4Fe-4S]-containing

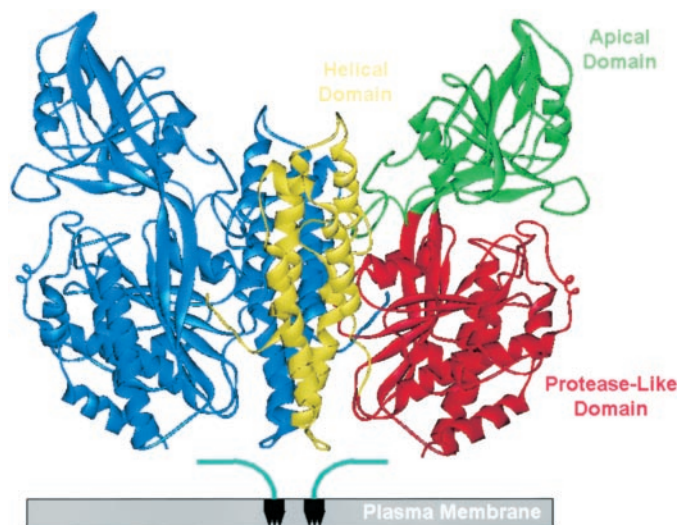


FIG 1. X-ray crystal structure of the ectodomain of the transferrin receptor 1. A ribbon diagram of the dimeric transferrin receptor 1 is organized as a butterfly-like shape. The transferrin receptor 1 monomer contains three distinct domains. The protease-like, apical and helical domains in one monomer are shown in red, green, and yellow, respectively, and the other monomer is in blue. The stalk region is shown in gray and connected to the putative membrane-spanning helices (Lawrence et al., 1999).

enzyme that catalyzes the isomerization of citrate to isocitrate. IRP1 has been regarded as a bifunctional "sensor" of iron, switching between RNA binding and enzymatic activities as aconitase depending on cellular iron status (Constable et al., 1992; Haile et al., 1992; Basilion et al., 1994). Under conditions of iron deficiency, IRP1 binds to the IREs in the 5'-untranslated region of ferritin mRNA (Hentze et al., 1987; Bhasker et al., 1993). This binding sterically prevents the recruitment of 43 S translation preinitiation complex and inhibits translation of ferritin. On the other hand, binding of IRP1 to IREs in the 3'-untranslated region of TfR1 mRNA increases mRNA stability and synthesis of TfR1 (Müllner and Kühn, 1988; Koeller et al., 1989; Müllner et al., 1989). In contrast, under high concentration of intracellular iron, IRP1 is enzymatically active and lacks RNA binding activity that leads to the degradation of TfR mRNA, whereas ferritin mRNA is translated efficiently (Koeller et al., 1989; Müllner et al., 1989).

IRP2 shares about 62% identity with IRP1 and, despite sequence similarities, it does not form a [4Fe-4S] cluster and consequently lacks aconitase activity (Guo et al., 1994). IRP2 binds specifically to all known mRNA IREs with an affinity equally as high as that of IRP1; however, their binding specificities to distinct sequences of iron-responsive elements differ significantly (Henderson et al., 1993; Guo et al., 1994). IRP2 differs from IRP1 in the mechanism by which iron levels are sensed (Guo et al., 1995; Iwai et al., 1995). IRP2 undergoes ubiquitination and proteasomal degradation in iron-replete cells, which is mediated by an iron-dependent oxidation mechanism requiring a unique 73-amino acid domain containing three cysteine residues (Iwai et al., 1995, 1998). IRP2 is induced following iron starvation through renewed synthesis of stable IRP2 protein and its inactivation by iron reflects degradation of IRP2 by a translation-dependent mechanism (Guo et al., 1994; Henderson and Kühn, 1995). It is likely that IRP1 and IRP2 perform distinct functions, probably by acting on different target genes. However, IRP1-deficient mice show no abnormalities in iron metabolism (Rouault and Klausner, 1997). Therefore, IRP2 might compensate for the function of IRP1.

The signals other than iron levels, such as nitric oxide and oxidative stress, can also regulate IRPs and modulate cellular iron metabolism (Drapier et al., 1993). It has been recently reported that the increased IRP activity induced by nitric oxide is one of the causes for the exercise-induced low iron status and high TfR expression (Qian et al., 1999b, 2001; Xiao and Qian, 2000; Qian, 2002). Several review papers have given detailed information (Hentze and Kühn, 1996; Aisen et al., 1999; Lieu et al., 2001). Briefly, nitric oxide and H₂O₂ produced from oxidative stress activate IRP1 by a cycloheximide-insensitive post-translational mechanism (Pantopoulos and Hentze, 1995), whereas IRP2 activation by nitric oxide requires de novo protein synthesis (Panto-

poulos et al., 1996). Nitric oxide regulates the binding of IRP1 by disassembling the iron-sulfur cluster or by acting as a cytoplasmic iron chelator, which results in a loss of aconitase activity (Drapier et al., 1993; Gardner et al., 1995; Ho et al., 2001; Bouton et al., 2002). The activation of IRP1 by H₂O₂ is dependent on extracellular signaling events (Pantopoulos et al., 1997; Pantopoulos and Hentze, 1998), suggesting that cluster disassembly alone cannot fully account for H₂O₂-induced IRP1 activation and that signaling pathways are involved.

C. Association of Transferrin Receptor 1 with the Hemochromatosis Protein HFE

The TfR binds two proteins critical for iron metabolism: Tf and HFE, the protein mutated in hereditary hemochromatosis (Pietrangelo, 2002; Waheed et al., 2002). Both the primary and crystal structure of HFE showed homology to class I major histocompatibility complex protein (Feder et al., 1996; Lebrón et al., 1998), that is composed of a heavy chain associated with β_2 -microglobulin (β_2 M). The mechanism by which HFE regulates iron uptake into the body is unknown. However, HFE was found to coprecipitate with TfR1 in tissue culture cells (Feder et al., 1998). The HFE-TfR1 complex can also be identified in human tissues such as the placenta and intestine (Parkkila et al., 1997; Waheed et al., 1999; Trinder et al., 2002). Upon forming an association complex in the endoplasmic reticulum, HFE cotrafficks with TfR through the Golgi reticulum network to reach the cell surface (Gross et al., 1998; Roy et al., 1999; Salter-Cid et al., 1999; Ramalingam et al., 2000). The wild-type HFE has been shown to negatively regulate Tf-mediated iron uptake in transfected cells (Parkkila et al., 1997; Gross et al., 1998; Roy et al., 1999; Schwake et al., 2002). This inhibitory effect is however attenuated in the Cys282Tyr mutant HFE (Feder et al., 1998; Okamoto, 2002; Schwake et al., 2002), suggesting that patients with HFE mutations might have pathological iron regulation, possibly via an altered TfR1-dependent iron metabolic pathway.

Similar to Fe-Tf, HFE binds tightly to soluble TfR1 at the cell surface pH 7.4 with K_d ca. 0.6 nM, with little or no binding at the acidic intracellular vesicles pH, suggesting that HFE dissociates from TfR1 in acidified endosomes (Lebrón et al., 1998). Both 2:1 and 2:2 TfR1/HFE stoichiometries have been observed (Lebrón et al., 1998; Bennett et al., 2000; West et al., 2001); however, the stoichiometry of TfR1/HFE on the cell membrane is not known. It has been demonstrated that membrane bound or soluble HFE binding to cell surface TfR1 results in a reduction in the affinity of TfR1 for Fe-Tf (Feder et al., 1998; Gross et al., 1998). This has been attributed to the formation of the ternary complex consisting of one Fe-Tf and one HFE bound to a TfR1 homodimer Tf (Lebrón et al., 1998, 1999). The HFE-TfR1

cocrystal structure (Fig. 2) reveals that HFE binds to the helical domain of TfR1, and a large surface area of interaction exists between HFE and TfR1 (Bennett et al., 2000). Binding of HFE induces conformational changes of TfR1. The backbone structure of the protease and apical domains remains unchanged in contrast to the helical domain in uncomplexed TfR1, which is displaced with respect to the other domains when compared with the structure of complexed TfR1. The movement alters the shape of the cleft between the helical and protease-like domains, and thus possibly alters the ability of Tf to bind to the TfR1 (Bennett et al., 2000). Mutation of five TfR1 residues at the HFE binding sites results in significant reductions in Tf binding affinity, which also support the idea that HFE and Tf compete for overlapping binding sites on TfR1 (West et al., 2001). However, the lower affinity of Tf for TfR1 by HFE is not a satisfactory explanation for the lower iron uptake since the concentration of $\text{Fe}_2\text{-Tf}$ in serum is very high (ca. 5 μM), and the binding of $\text{Fe}_2\text{-Tf}$ to its receptor is saturated even in the presence of HFE. A recent study showed that overexpression of HFE without overexpression of $\beta_2\text{M}$ results in a decrease in TfR1-dependent iron uptake in transfected Chinese hamster ovary cell lines, whereas overexpression of both HFE and $\beta_2\text{M}$ results in an increase in TfR1-dependent iron uptake and increased iron levels in the cells (Waheed et al., 2002). There is also a hypothesis that HFE has two mutual activities in cell, i.e., inhibition of uptake or inhibition of release of iron, and the balance between Tf saturation and TfR1 concentrations determined which of these functions predominates (Townsend and Drakesmith, 2002). Functional significance of the association of HFE with the TfR1 and the critical regulatory role of HFE in iron metabolism remain unveiled.

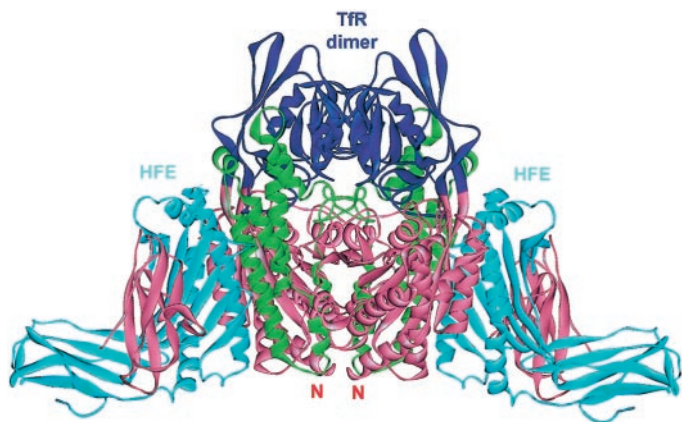


FIG 2. Crystal structure of the complex of HFE and transferrin receptor 1. A ribbon diagram of the complex reveals that two HFE molecules grasp each side of a transferrin receptor 1 homodimer on the same membrane, giving rise to a 2:2 stoichiometry. Noticeably, the complexation of HFE appears to induce conformational changes in transferrin receptor 1, thus to influence the function of transferrin receptor 1 in terms of transferrin binding and internalization (Bennett et al., 2000).

D. Second Transferrin-Binding Protein: Transferrin Receptor 2

A new TfR-like family member, transferrin receptor 2 (TfR2), has been cloned and sequenced (Kawabata et al., 1999). The TfR2 gene is located on chromosome 7q22 and gives rise to two transcripts of approximately 2.9 and 2.5 kb in length (Kawabata et al., 1999). Amino acid sequence analysis reveals that, like TfR1, TfR2 is a type II transmembrane glycoprotein that shares a 45% identity and 66% similarity in its extracellular domain with TfR1. The α -transcript product is primarily expressed in the livers of humans and mice (Kawabata et al., 1999; Fleming et al., 2000) and the TfR2 β -transcript distributed widely but is expressed at low levels (Kawabata et al., 1999). Sequence analysis of TfR2 coding and noncoding region reveals that TfR2 does not possess IRE (Kawabata et al., 1999). Therefore, it is likely that expression of TfR2 is not regulated by IRP-mediated feedback regulatory mechanism in response to cellular iron status. Instead, it may be regulated by a different mechanism, probably related to the cell cycle or cellular proliferation status (Kawabata et al., 2000). TfR2 has a similar function to TfR1 with respect to Tf binding and Tf-mediated iron uptake. Both TfR1 and TfR2 interact with Tf in a pH dependent manner; apo-Tf binds to these receptors only at acidic pH and holo-Tf binds at neutral or higher pH (Kawabata et al., 2000). However, the affinity of TfR2 for iron-loaded Tf is 25-fold lower than that of TfR1 for Tf (West et al., 2000).

The expression pattern for TfR2 is distinct from that for TfR1. It has been shown that during liver development, TfR2 was up-regulated and TfR1 was down-regulated; during erythrocytic differentiation of murine erythroleukemia cells induced by dimethylsulfoxide, expression of TfR1 increased, whereas TfR2 decreased (Kawabata et al., 2001a). Therefore, TfR2 appears to serve unique functions involved in iron metabolism, hepatocyte function, and erythrocytic differentiation. In addition, high levels of TfR2 expression were also found in the erythroid cell lines including erythroid leukemia cell line. Levels of expression of TfR2- α mRNA were found to be significantly higher in erythroleukemia marrow samples than in nonmalignant control marrow samples, suggesting that TfR2- α may be a useful marker of early erythroid precursor cells (Kawabata et al., 2001b). The clinical significance of TfR2- α expression in leukemia cells remains to be determined. Mutations in TfR2 have been identified as the cause of a form of hemochromatosis that is not linked to the mutation of HFE (Camaschella et al., 2000; Roetto et al., 2001), which suggests that TfR2 is associated with iron overload and offers a tool for molecular diagnosis of non-HFE related disorders.

The reason for the existence of two TfRs is still not fully understood. The high level of TfR2 expression in the liver suggests a particular role for this receptor,

possibly TfR2 contributes substantially to the liver's ability to capture and store iron. In addition, a possibility has recently been suggested that TfR2 might play an important role in modulation of hepcidin expression, thus involving the regulation of dietary iron absorption (Nicolas et al., 2001; Fleming and Sly, 2001, 2002). Deficiency of TfR2 (Camaschella et al., 2000; Roetto et al., 2001; Girelli et al., 2002) has been demonstrated to cause an hereditary hemochromatosis-like phenotype, implicating TfR2 as a participant in iron homeostasis. On the other hand, the observation that the TfR1 knockout mutation in the mouse leads to an embryonic lethal phenotype demonstrated that TfR2 couldn't fully compensate for the functions of TfR1 (Levy et al., 1999).

IV. Transferrin Receptor-Mediated Iron Uptake

Iron is vital for almost all living organisms. However, iron concentrations in body tissues must be tightly regulated because excessive iron leads to tissue damage as a result of the formation of free radicals. Acquisition of iron is a challenge in which many proteins participate to ensure that iron uptake is sufficient and appropriate to the needs of cells, but the total number of proteins involved in mammalian iron metabolism is unknown (Andrews, 1999). Recently, several new genes have been identified, which provide insights into the mechanism of iron absorption by the enterocytes of the duodenal mucosa (Jiang and Qian, 2001; Ke and Qian, 2002). Fe^{3+} in food is reduced to Fe^{2+} by duodenal cytochrome *b* (McKie et al., 2001) and then absorbed into the enterocyte by an iron transporter protein DMT1 (Fleming et al., 1997; Gunshin et al., 1997). Iron subsequently crosses the enterocyte and is exported from its basolateral surface by another iron transporter, ferroportin 1 (Donovan et al., 2000). In the export process the iron is reoxidized to Fe^{3+} by hephaestin (Vulpe et al., 1999) and bound to serum Tf (Aisen, 1998). In humans, failure to maintain appropriate levels of iron is a feature of iron-deficiency anemia, hereditary hemochromatosis, and even certain neurodegenerative diseases (Smith et al., 1997; Qian et al., 1997a; Qian and Wang, 1998; Andrews, 1999; Qian and Shen, 2001). Abnormally high levels of iron have been found in some regions of the brain in neurodegenerative disorders (Qian and Wang, 1998; Aisen et al., 1999; Qian and Ke, 2001), although it is not clear whether iron accumulation in the brain is an initial event that causes neuronal death or is a consequence of the disease process. It is likely that misregulation of iron metabolism is important in the pathophysiology of certain neurodegenerative diseases (Qian and Wang, 1998; Qian and Shen, 2001; Rouault, 2001).

A. Transferrin-Bound Iron Uptake by Cells

Cells take up iron by using a variety of mechanisms. In high organisms, one principal pathway of cellular iron acquisition is by the receptor-mediated uptake of

transferrin-bound iron, which is one of the best understood processes in cell biology. Several review papers have given a detailed description (Qian and Tang, 1995; Morgan, 1996; Qian et al., 1997b; Aisen, 1998; Andrews, 1999, 2000; Lieu et al., 2001). Figure 3 shows the current model of iron uptake from Tf via TfR1-mediated endocytosis. Briefly, the process is triggered by the binding of $\text{Fe}_2\text{-Tf}$ to a specific cell-surface TfR1 (Morgan and Laurell, 1963; Morgan and Appleton, 1969). After endocytosis via clathrin-coated pits, which bud from the plasma membrane as membrane bound vesicles or endosomes, the $\text{Fe}_2\text{-Tf-TfR1}$ complex is routed into the endosomal compartment. Upon maturation and loss of the clathrin coat, the endosome becomes competent to pump protons in a process energized by ATPase, and the endosomal lumen is rapidly acidified to a pH of about 5.5 (Dautry-Varsat et al., 1983; Klausner et al., 1983; Paterson et al., 1984). At this pH, the binding of iron to Tf is weakened, leading to iron release from the protein. The free Fe^{3+} released to endosomes is reduced to Fe^{2+} on the *cis*-side of the endosomal membrane probably mediated by oxidoreductase (Núñez et al., 1990). Fe^{2+} is subsequently transported out of the Tf cycle endosome by the divalent metal transporter DMT1, i.e., from the endosomal membrane to the cytosol (Fleming et al., 1998; Tabuchi et al., 2000). Once in the cytosol, iron is utilized as a cofactor for aconitase, the cytochromes, RNA reductase, and heme, or stored as ferritin. After release of iron into the endosome, the resultant apo-Tf-TfR1 complex is then recruited through exocytic vesicles back to the cell surface. At extracellular physiological pH, apo-Tf dissociates from its receptor due to its low affinity at pH 7.4, is released into the circulation, and reutilized (Morgan,

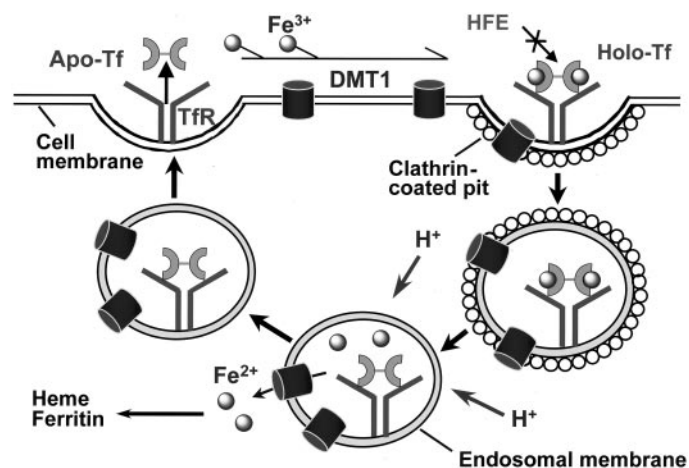


FIG 3. The cycle of transferrin and transferrin receptor 1-mediated cellular iron uptake. Differic transferrin (holo-transferrin) binds to transferrin receptor 1 on the cell surface. The resulting complex is internalized and acidified through the action of a proton pump. Iron is subsequently released from transferrin and transported out of endosomes via the divalent metal transporter DMT1. HFE appears to inhibit transferrin receptor 1 endocytosis, probably through binding to transferrin receptor 1. Apo-transferrin and transferrin receptor 1 both returned to the cell surface, where they dissociate at neutral pH, and both participate in another round cycle of iron uptake. Intracellular iron is either incorporated into heme or stored in ferritin.

1996, 2001; Qian et al., 1997b). ATP-mediated energy is necessary for sustaining TfR-mediated endocytosis and recycling (Podbilewicz and Mellman, 1990; Schmid and Smythe, 1991).

It has been shown that the number of receptors displayed on the cell surface is proportional to iron uptake and that iron deficiency induces TfR1 gene expression (Aisen, 1998), which implies the significance of TfRs in iron uptake. Furthermore, surface display of TfR1 is affected by its total cellular concentration, as well as its distribution and rate of recycling between the cell surface and cell interior. The efficiency of TfR1 function is also influenced by other proteins, including SFT (stimulator of iron transport) and HFE. SFT stimulates iron uptake by both Tf and non-Tf pathways (Gutierrez et al., 1997), whereas HFE appears to negatively modulate Tf-dependent iron uptake (Parkkila et al., 1997; Roy et al., 1999).

B. Iron Transport Across the Blood-Brain Barrier

To date, the mechanisms of iron transport across the blood-brain barrier (BBB) have not been completely clarified. The accumulated evidence suggests that the Tf and TfR pathway may be the major route of iron transport across the luminal membrane of the capillary endothelium (Bradbury, 1997; Moos and Morgan, 1998, 2000; Malecki et al., 1999), and that iron, possibly in the form of ferrous iron, crosses the abluminal membrane and enters into the brain, although the molecular events of this process are unknown (Bradbury, 1997; Moos and Morgan, 1998). The evidence shows that the uptake of Tf-bound iron by TfR-mediated endocytosis from the blood into the cerebral endothelial cells is no different in nature from the uptake into other cell types (Bradbury, 1997). As found in other cells, this process also includes several steps: binding, endocytosis, acidification and dissociation, and translocation of iron across the endosomal membrane. Most of the Tf will then return to the luminal membrane with TfR, whereas the iron then crosses the abluminal membrane by an undetermined mechanism (Bradbury, 1997; Moos and Morgan, 1998). As mentioned before, recent studies have shown that ferroportin 1/hephaestin and/or hephaestin-independent iron export systems might play a key role in ferrous iron transport across basal membrane of enterocytes in the gut (Vulpe et al., 1999; Donovan et al., 2000; Kaplan and Kushner, 2000). This process is very similar to what occurred in the BBB cells (capillary endothelium) (Qian and Shen, 2001). Because the form of iron transport across this membrane might be ferrous iron (Bradbury, 1997; Moos and Morgan, 1998), therefore, a ferroxidase such as hephaestin (or ceruloplasmin) might be necessary for ferrous iron to be oxidized to ferric iron, so that iron, after crossing the basolateral membrane of the BBB cells, could be carried away by Tf (Ke and Qian, 2001). Based on the similarity of the transport form of iron across the basolateral membranes (both are ferrous

iron), and the existence of ferroportin 1 and hephaestin in the brain (Jiang et al., 2002), it is possible that ferroportin 1/hephaestin or ferroportin 1/ceruloplasmin system might play a role in iron transport across the BBB cells. Another proposed mechanism involved in ferrous iron transport across abluminal membrane is the role of astrocytes. The astrocytes probably have the ability to take up ferrous iron from endothelial cells through their end feet processes on the capillary endothelia (Malecki et al., 1999; Oshiro et al., 2000). In addition to Tf/TfR pathway, it has been suggested that the lactoferrin receptor/lactoferrin and GPI-anchored p97/secreted p97 pathways might play a role in iron transport across the BBB (Faucheux et al., 1995; Qian and Wang, 1998; Malecki et al., 1999). It is also possible that a small amount of iron might cross the BBB in the form of intact Tf/Fe complex by receptor-mediated transcytosis (Moos and Morgan, 1998) (Fig. 4). After the iron has been transported across the BBB, it is likely to bind quickly to the Tf that is secreted from the oligodendrocytes and choroid plexus epithelial cells (Bradbury, 1997; Moos and Morgan, 1998) or other transporters and then transported to where iron is needed (Qian and Shen, 2001).

V. Transferrin As a Metallodrug Mediator

A. Complexation of Metal-Based Drugs with Transferrin

The transferrins are primarily iron-binding proteins, but in human serum, Tf is only about 30% saturated with iron, so there is a potential capacity for binding to other metal ions that enter the body. Indeed, over 30 metal ions have been reported to bind to Tf with either carbonate, oxalate, or other carboxylates as synergistic anions, although Fe^{3+} has a higher affinity than any other metal ion for which the binding constant has been determined (Aisen, 1998; Sun et al., 1999). Such binding may play an important role in the transport and delivery of medical diagnostic radioisotopes such as $^{67}\text{Ga}^{3+}$ and $^{111}\text{In}^{3+}$ (Harris and Pecoraro, 1983; Ward and Taylor, 1988; Harris et al., 1994; Bernstein, 1998) and therapeutic metal ions such as Bi^{3+} (Li et al., 1996a; Sun et al., 2001; Zhang et al., 2001), Ru^{3+} (Kratz et al., 1994; Smith et al., 1996) and Ti^{4+} (Sun et al., 1998a; Messori et al., 1999).

Electronic absorption spectroscopy is frequently used to detect metal binding to the specific iron sites of transferrins. Apo-transferrin (apo-Tf) is a colorless protein with an intense ultraviolet absorption near 280 nm with ϵ_{278} 93,000 $\text{M}^{-1} \text{cm}^{-1}$, attributable to π - π^* transitions of the aromatic amino acids tyrosine, tryptophan, and phenylalanine. The binding of metal ions to the phenolic groups of the tyrosine residues in the specific metal binding sites of apo-Tf leads to the production of two new absorption bands centered at ca. 240 nm and ca. 295 nm in the UV-difference spectra. This has been widely exploited for metal titration and thermodynamic studies.

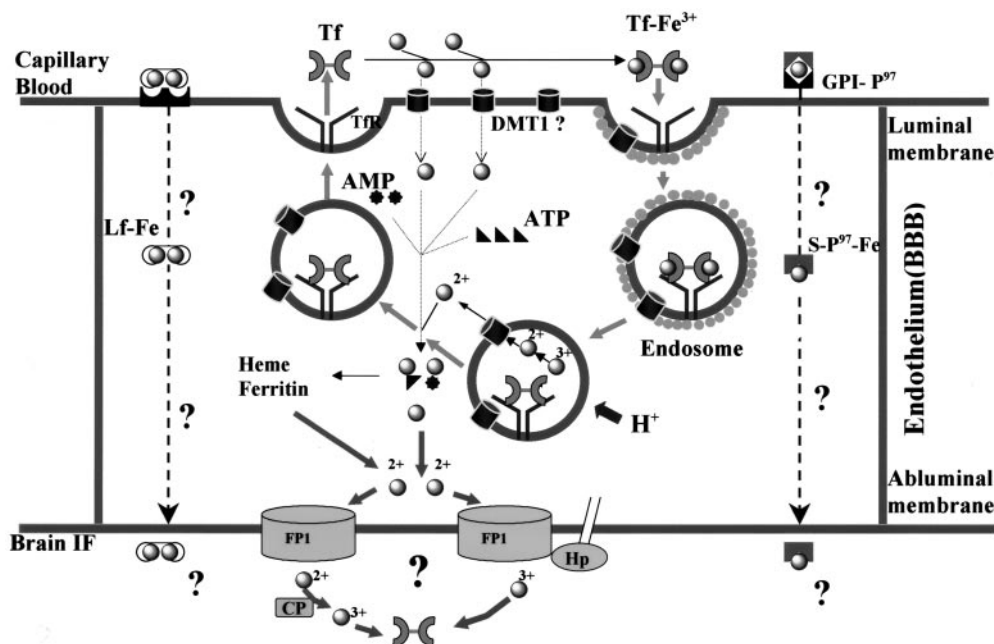


FIG 4. The role of Tf and TfR in iron transport across the BBB. The Tf/TfR pathway may be the major route of iron transport across the luminal membrane of the BBB. The Tf-Fe (transferrin-bound iron) uptake by endothelial cells is no different in nature from the uptake into other cell types. DMT1 might play a role in translocation of iron from endosome to cytosol. Then iron (Fe^{2+}) crosses the abluminal membrane probably via FP1 (ferroportin 1)/HP (hephaestin) and/or HP-independent export systems. LfR/Lf and GPI-P⁹⁷/S-P⁹⁷ pathways might also involve iron transport across the BBB. (GPI-P⁹⁷, GPI-anchored P⁹⁷; Lf/LfR, lactoferrin/lactoferrin receptor; S-P⁹⁷, secreted P⁹⁷; ?, to be confirmed).

Typical difference spectra are shown in Fig. 5, in which two new bands centered at 241 and 295 nm appear and increase in intensity with time after addition of 2 mol Eq of Bi^{3+} as $[\text{Bi}(\text{Hcit})]$, indicative of a slow complexation of Bi^{3+} in the specific binding sites of apo-Tf. When transition metal ions bind to apo-Tf, there are often additional intense tyrosinate-to-metal charge transfer (LMCT) bands in the visible region of the spectrum (400–500 nm; ϵ ca. $4\text{--}9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) that are also diagnostic of site-specific binding. For example, the Fe^{3+} complex is orange-red with a band at ca. 465 nm, the Cu^{2+} and Co^{3+} complexes are yellow, and the Mn^{3+} complex is brown (Aisen et al., 1969). Electronic absorption spectroscopy also allows determination of the strength of metal binding to Tf (Sun et al., 1999). The strength of binding of metal ions has been found to correlate well with metal ion acidity, and the most readily hydrolyzed (most acidic) metal ions bind most strongly to transferrin (Li et al., 1996b; Sun et al., 1997a). This provides a basis for the prediction of unknown stability constants for metal-Tf complexes and allows the discovery of new metal ion (e.g., Ti^{4+}) binding to Tf (Sun et al., 1998a). Table 1 lists the binding constants for therapeutic metal ions with Tf.

B. Structural Studies of the Complexes of Therapeutic Metal Ions with Transferrin

Iron binding to Tf induces protein structure changes from an open to a closed conformation, which is thought to be crucial for receptor recognition. Therefore it is important to study the structural changes induced by

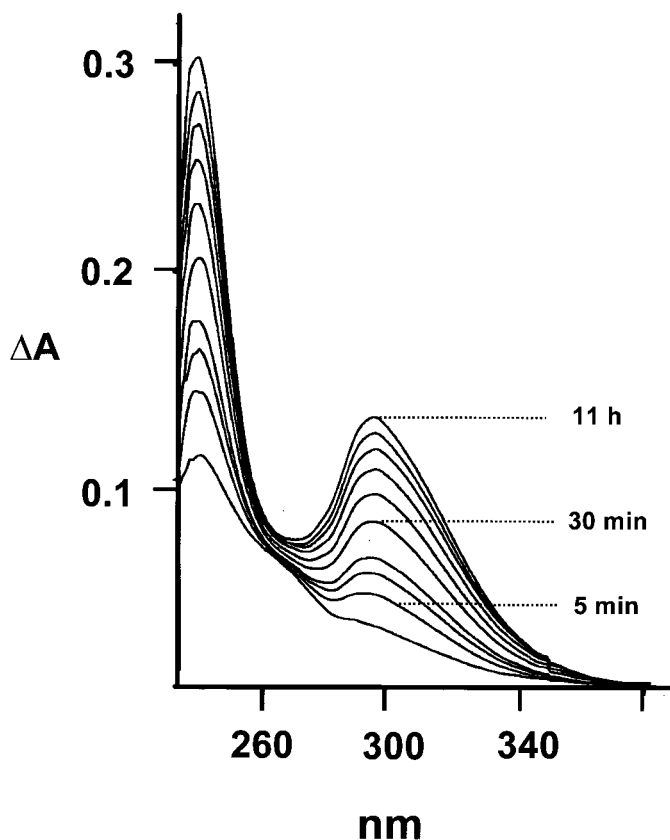


FIG 5. Detection of metal binding to transferrin via UV different spectroscopy. $\text{Bi}(\text{III})$ citrate (2 mol Eq) is added to apo-transferrin solution at pH 7.4, 5 mM bicarbonate. The slow uptake of Bi^{3+} by transferrin is evidenced by the appearance of two bands at 241 and 295 nm due to deprotonation of phenol groups of Tyr residues of transferrin (Li et al., 1996a).

TABLE 1
Stability constants for the therapeutic metal ions binding to Tf (log K) and the uptake of these metals in various cell lines

Metal Ions	Log K_1	Log K_2	Cell Lines Used	Special Remarks	References
Ga^{3+}	19.7	18.8	Human leukemic HL60	TfR-dependent and TfR-independent mechanism, Ga-Tf inhibits iron incorporation	Harris and Pecoraro, 1983; Chitambar et al., 1987, 1988; Chitambar and Zivkovic-Gilgenbach, 1990
			Small cell lung Cancer cells	Tf and TfR are important	Weiner et al., 1996
			EMT-6 sarcoma	TfR-mediated uptake	Larson et al., 1979
			Murine tumor cell	Exogenous Tf stimulated Ga^{3+} uptake significantly	Harris and Sephton, 1977
In^{3+}	18.5	16.6	Lymphomas	TfR pathway	Habeshaw et al., 1983
			Human and rat reticulocytes	In-Tf binds to cell membrane, but minimal transfer into the cell	Beamish and Brown, 1974; Harris et al., 1994
			Human leukemia cell line	Cellular proliferation inhibited by In-Tf and TfR expression increased	Moran and Seligman, 1989
Bi^{3+}	19.4	18.6	BeWo placental cancer cells; IEC-6 rat intestinal cells	Recognized by cells, interfere with iron uptake	Li et al., 1996a; Guo et al., 2000; Zhang et al., 2001
Ti^{4+}			BeWo placental cancer cells	TfR-mediated uptake by cells	Guo et al., 2000
Ru^{3+}			Jurkat T_{ag} cell; tumor and abscess; human colon cancer cell	Ru^{3+} toxicity increased; Ru-Tf in localization tumor and abscess	Som et al., 1983; Frasca et al., 2001
			SW707	Tf as a carrier for Ru^{3+}	Kratz et al., 1996

other metal ions. Although many crystal structures are available for either apo-Tf or holo-Tf, there appears very few for other metal-Tf (Shongwe et al., 1992; Smith et al., 1996). X-Ray crystallographic studies of human lactoferrin have demonstrated that Ru^{3+} coordinates directly to the imidazole nitrogen of His253, one of the Fe^{3+} ligands in the iron binding cleft of the N-lobe, with displacement of a chloride ligand. At least one indazole ligand remains coordinated to the Ru^{3+} (Smith et al., 1996). However, this study could not provide whether the protein would form the "closed" structure with Ru complexes. Modeling by superposition of normal closed structure suggests that certain Ru^{3+} complexes may allow closure of the protein (Smith et al., 1996). Small angle X-ray scattering has also been used to provide direct structural information on the conformational changes induced by metal ions. It was shown that In^{3+} induced the same domain closure as Fe^{3+} (Grossmann et al., 1993b).

Isotopic labeling of Tf can provide assignments of resonances for specific amino acid residues of Tf, which enhances the usefulness of NMR spectroscopy in exploring conformational changes in the protein. By means of two-dimensional NMR and single site mutations, the $\text{S-}^{13}\text{CH}_3$ resonances of all the five Met residues of the N-lobe and nine Met residues of intact Tf have been tentatively assigned (Beatty et al., 1996; He et al., 1999b). The Met residues are well spread throughout the protein and occupy several environments. Some of the Met resonances (e.g., Met464 and Met109) are sensitive to metal binding even though these residues are far away from the metal binding site (>10 Å). Similar

changes in $^1\text{H}/^{13}\text{C}$ shifts of Met resonances are observed for Fe^{3+} , Ga^{3+} , and Bi^{3+} indicating that they induce similar conformational changes (Sun et al., 1998b). Ti^{4+} also induces a similar structural change as Fe^{3+} (Guo et al., 2000). This approach provides a useful technique for probe protein conformational changes induced by metals under biologically relevant conditions (Sun et al., 2001).

C. Cellular Uptake of Therapeutic Metal Ions via Transferrin Receptor-Mediated Endocytosis

There is increasing interest in the use of metal-containing compounds in medicine, such as the use of platinum complexes in cancer chemotherapy, gold compounds in the treatment of arthritis, gallium and indium in diagnosis and radiotherapy, and bismuth in anti-ulcer medication (Abrams and Murrer, 1993). One concern is how we can ensure that a metal-based therapeutic or diagnostic agent reaches its target site. One way is to incorporate features that are recognized specifically by the target. We can seek to use natural recognition mechanisms such as the Tf/TfR recognition system. The natural Tf cycle for the delivery of Fe^{3+} to cells offers an attractive system for strategies of drug delivery and targeting since TfR can bind strongly to a range of other metal ions apart from Fe^{3+} , and many heterometal-Tf complexes are still recognized by the TfR.

1. Ga^{3+} and In^{3+} . The mechanisms by which Ga^{3+} is transported into target sites are of fundamental importance since gallium compounds have been used extensively both in the diagnosis and the treatment of human cancers (Tsuchiya et al., 1992; Bernstein, 1998). It has been shown previously that Ga^{3+} binds to Tf in the

specific Fe^{3+} binding sites with a similar affinity, attributed to the similarity between these two metal ions (Harris and Pecoraro, 1983). In vivo studies using ^{67}Ga find that all gallium in blood is present in plasma (with traces in leukocytes) and is tightly bound to Tf (Clausen et al., 1974). There has been a quite controversy about whether ^{67}Ga uptake is a Tf-independent or -dependent process and whether tumors and normal tissues differ in the mechanism of uptake. Early studies have shown that the uptake of ^{67}Ga by cultured murine tumor cells can be significantly stimulated by the addition of exogenous transferrin to the tissue culture medium (Harris and Sephton, 1977). In contrast, a decrease in ^{67}Ga uptake by certain tumor cells has been noticed following the addition of Tf to the incubation medium (Vallabhajosula et al., 1981). The uptake study of $^{67}\text{Ga}^{3+}$ by human leukemic cell line HL60 demonstrated that both a transferrin receptor-dependent and a Tf-independent mechanism exist. HL60 cells incorporated about 1% of the Ga^{3+} dose over 6 h in the absence of Tf. However, the presence of Tf could increase cellular Ga^{3+} uptake approximately 10-fold (Chitambar and Zivkovic, 1987). Anti-Tf receptor monoclonal antibody inhibited Ga^{3+} uptake, and decrease in the density of cellular TfR led to corresponding decreases in the Tf-dependent uptake of Ga^{3+} (Chitambar and Zivkovic, 1987). Cell surface-bound $\text{Ga}_2\text{-Tf}$ displayed similar kinetics as $\text{Fe}_2\text{-Tf}$ during the first 10-min uptake, suggesting that the initial internalization of $\text{Ga}_2\text{-Tf}$ closely resembled that of $\text{Fe}_2\text{-Tf}$ (Chitambar and Zivkovic-Gilgenbach, 1990). However, unlike ^{59}Fe , a small fraction of internalized ^{67}Ga was released from cells by an unknown reason. Ammonium chloride inhibited the internalization of both ^{67}Ga and ^{59}Fe , indicating that $^{67}\text{Ga}\text{-Tf}$ uptake by HL60 cells involves initial internalization into acidic receptosome and followed by dissociation of ^{67}Ga and Tf and subsequent trafficking of each to separate compartments (Chitambar and Zivkovic-Gilgenbach, 1990). $\text{Ga}_2\text{-Tf}$ disrupts TfR-mediated cellular uptake of iron as results of inhibition of the iron-containing M2 subunit of ribonucleotide reductase (Chitambar et al., 1988, 1991). Transferrin-enhanced uptake of ^{67}Ga was also found in other cell lines (Table 1). Therefore, it can be concluded that the TfR pathway is of primary importance in the incorporation of Ga^{3+} into the cytoplasm of cells displaying this receptor. However, ^{67}Ga can also enter tumors and other cells by a Tf-independent mechanism, which is probably also used by iron (Chitambar and Zivkovic, 1987; Weiner et al., 1996); this becomes apparent when Tf is in short supply or saturated with iron or other metal ions (Sohn et al., 1993).

Similar to Ga^{3+} , the In^{3+} has also been investigated intensively because of the widespread interest in its use in radiopharmaceuticals. In^{3+} binds to transferrin strongly but slowly compared with Ga^{3+} (Harris et al., 1994). When indium is injected either as an acidic solution or as a weak chelate such as citrate, more than 95%

binds to macromolecular ligands, which appear to be Tf (Tsan et al., 1980; Raijmakers et al., 1992; Hulle et al., 2001). The binding affinities of $\text{In}_2\text{-Tf}$ and $\text{Fe}_2\text{-Tf}$ to the Tf receptors on reticulocytes are very similar (Beamish and Brown, 1974). The uptake study of ^{111}In and ^{59}Fe bound to Tf by human and rat reticulocytes showed that uptake of In^{3+} from human and rat serum was 30% and 12% that of Fe^{3+} after 30 min of incubation, and this process was temperature-dependent (Beamish and Brown, 1974). Washed reticulocytes, previously incubated for 30 min with either ^{59}Fe or ^{111}In bound to serum were incubated in unlabeled serum. It was found that up to 85% of the ^{111}In label and less than 10% of the ^{59}Fe on the reticulocytes were released on reincubation, indicating that in contrast to ^{59}Fe , the majority of the ^{111}In label remained membrane bound (Beamish and Brown, 1974). Unlike iron, there is minimal transfer of In^{3+} into the cell or incorporation into heme (Beamish and Brown, 1974).

2. Bi^{3+} , Ti^{3+} and Ru^{3+} . Bi^{3+} complexes are in widespread use in the treatment of ulcers (Sun et al., 1997b; Sun and Sadler, 1998; Briand and Burford, 1999; Sadler et al., 1999); Ru^{3+} compounds are potential anticancer agents, which are often active against metastases but not against the primary tumors (Kratz et al., 1994; Clarke et al., 1999); Ti^{4+} complexes have been shown to exhibit high antitumor activities against a wide range of murine and human tumors with less toxic side effects than cisplatin (Köpf-Maier and Köpf, 1987; Harding and Mokdsi, 2000). There are two titanium complexes, titanocene dichloride and budotitane, now in clinical trials (Köpf-Maier and Köpf, 1987; Keppler et al., 1991). All these metal ions have been found binding to Tf strongly in the specific iron sites (Table 1). Therefore, Tf may act as a carrier to deliver these therapeutic metal ions into the cells. Cell uptake experiments showed that $\text{Ti}_2\text{-Tf}$ and $\text{Bi}_2\text{-Tf}$ can block both membrane binding and cellular uptake of $\text{Fe}_2\text{-Tf}$ (Guo et al., 2000). These experiments provide evidence that both bismuth and titanium are likely transported via a similar mechanism as iron, i.e., TfR-mediated endocytosis. Recognition of $\text{Bi}_2\text{-lactoferrin}$ by IEC-6 rat intestinal cells (Zhang et al., 2001) may also have implications for bismuth antimicrobial action. It is likely that bismuth may block the pathway of iron transport into the bacteria and cuts iron supply required by the bacteria for its growth. A recent study showed that Ti^{4+} does not bind strongly to DNA bases at physiological pH but forms strong complexes with nucleotides only at low pH values (below 5) (Guo and Sadler, 2000). Therefore, Tf may serve as a carrier to deliver titanium complexes to tumor cells and to prevent hydrolysis of Ti^{4+} complexes at neutral pH. Titanium is subsequently released as a result of acidic microenvironment in tumors than in normal tissue (Yamagata and Tannock, 1996), and targets DNA. Ru^{3+} complexes were reported bound to both albumin and Tf with an 80% portion binding to albumin and the remainder to the

latter (Messori et al., 2000; Frasca et al., 2001). Injection of Ru^{3+} -Tf resulted in high tumor uptake of the metal (Som et al., 1983; Ando et al., 1988; Srivastava et al., 1989), which suggests that Tf uptake appears to be the more important mode of transport of Ru^{3+} anticancer complexes to the tumor. The Ru_2 -Tf exhibits a significantly higher antitumor activity against human colon cancer cells than the albumin-bound complex or the Ru^{3+} complex itself (Kratz et al., 1994, 1996), probably attributed to the Tf-mediated uptake mechanism, which may lower ruthenium toxicity by preventing it from other binding or uptake until it has been delivered to the cells.

VI. Transferrin Conjugates in Site-Specific Drug Delivery

A. General Methods of Preparation of the Conjugates

1. *Chemical Linkage.* Various therapeutic agents have been chemically linked to Tf. Several studies have been reported to link doxorubicin with Tf via the formation of a Schiff base (Yeh and Faulk, 1984; Barabas et al., 1992; Sizensky et al., 1992; Bérczi et al., 1993a,b; Singh et al., 1998). Glutaraldehyde was frequently used for this purpose. Briefly, certain amounts of Tf and doxorubicin both dissolved in 150 mM NaCl were directly mixed and followed by the addition of glutaraldehyde (in 150 mM NaCl) dropwise. The coupling procedure was stopped by the addition of ethanolamine. The conjugate was subjected subsequently to purification and characterization. The conjugates prepared in such a way were found to exert cytotoxicity (Yeh and Faulk, 1984; Barabas et al., 1992; Bérczi et al., 1993a,b).

Although direct coupling methods are easy to carry out, they have some disadvantages that polymeric products are likely to be formed during the preparation, and the resulting conjugates are chemically poorly defined with respect to the chemical link between drugs and carrier proteins (Kratz and Beyer, 1998b; Singh, 1999). A new coupling approach has been attempted, in which the stability of the bond between the Tf and the drug can be finely tuned (Kratz et al., 1998a). This was achieved by synthesis of the first derivatizing the drug with a spacer group, such as maleimide spacer, and then attaching the drug derivative to the carrier protein (e.g., Tf). In this way, the bond between the drug and the spacer can act as a cleavage site, allowing the drug to be released inside the cells.

2. *Protein Engineering.* A novel alternative approach to using the Tf uptake pathway for cellular delivery of therapeutic agents is to incorporate the drug into the structure of Tf using recombinant protein engineering (Ali et al., 1999a,b). A therapeutic peptide sequence cleavable by the human immunodeficiency virus type 1 protease has been inserted into various regions of human serum Tf by protein engineering techniques. These insertions were cloned and expressed using a baculovi-

rus expression vector system. The results showed that mutant proteins retained the native Tf function and that the inserted peptide sequence was surface-exposed. Most importantly, two of the mutants could be cleaved by human immunodeficiency virus-1 protease (Ali et al., 1999a,b). In another study, Tf was fused to mouse-human chimeric IgG3 at different positions by protein engineering, and the resulting fusion protein was found to be able to cross the BBB and to target the brain (Shin et al., 1995). These studies have demonstrated the potential of Tf not only as a carrier protein for site-specific drug delivery, but also for developing new therapeutic agents for a broad spectrum of diseases in the future.

B. Cellular Uptake and Efficacy of the Conjugates

1. *Transferrin-Doxorubicin.* Although doxorubicin (Adriamycin) is an effective and widely used cancer chemotherapeutic agent; cardiotoxicity and emergence of resistance tumor cell lines significantly limit its utility in clinical practice (Kovar et al., 2002). Various approaches have been devised to circumvent these limitations, among which is the attachment of cytotoxic drugs to suitable carrier proteins, such as Tf, that accumulate in tumor tissue. The Tf-doxorubicin conjugate has been shown to exhibit greatly increased cytotoxicity relative to unconjugated doxorubicin toward a variety of culture cell lines (Table 2). Cellular uptake experiments revealed that free doxorubicin at concentrations below 1×10^{-7} M had little effect on K-562 cell, while Tf-doxorubicin conjugate inhibited 75% of cellular activity. When normal peripheral blood mononuclear cells were tested against the conjugate, the 50% inhibitory concentration was found to be 1.4 to 1.7×10^{-6} M, at which concentration over 85% of K-562 cells were inhibited (Sizensky et al., 1992). In another cytotoxicity assays, K-562 cells were exposed to doxorubicin or Tf-doxorubicin and cultured for 16 to 18 h. It was found that at a concentration of $0.05 \mu\text{M}$, 37% inhibition of K-562 observed for the conjugate compared with 5% for the free drug (Bérczi et al., 1993b). In vivo studies showed that the life span of tumor-bearing mice was significantly increased when they were treated with Tf-doxorubicin conjugate (increase in life span 69% versus 39% with doxorubicin); although no long-term survivors was observed, the tumor burden with conjugate-treated mice was much smaller compared with free doxorubicin-treated mice (Singh et al., 1998).

Significantly, Tf-doxorubicin conjugate exhibited cytotoxic effects in many multidrug-resistant cells. The Tf-doxorubicin conjugate was 4 to 5 times more potent than free drug in doxorubicin-sensitive tumor cell lines such as HL60, Hep2 in vitro, whereas 5 and 10 times more potent in resistant cell lines (Singh et al., 1998). In another resistant cell line L292, the IC_{50} for the Tf-doxorubicin conjugate was found to be 130-fold lower than that of free drug (Lai et al., 1998). The cytotoxicity was also compared between the conjugate of Tf-doxoru-

TABLE 2
Conjugates of therapeutic agents with Tf

Therapeutics	Linker Strategy	Biological Evaluation	Special Remarks	References
Doxorubicin	Phenylacetyl hydrazone	MDA-MB-435	Slightly improved activity and significantly reduced toxicity	Kratz et al., 2000
	Maleimide spacer	MDA-MB-468 and LXFL 592; L292	Amide or acid-sensitive link	Kratz et al., 1998a
	Glutaraldehyde	K562	Mechanistic studies	Lai et al., 1998
		HeLa		Bérczi et al., 1993a
		K562		Sun et al., 1992
		K562		Barabas et al., 1992
		H-Meso, HL60, Hep2	Plasma membrane as the target	Barabas et al., 1991
		NuGC4, SW403, tumor-bearing mice	More potent against resistant cell lines	Singh et al., 1998
		KB-3-1, KB-8-5, KB-C1, KB-V1, MCF-7	Prolong lifespan of the mice effectively against multidrug-resistant tumor cells	Fritzer et al., 1996
		Resistant K562	Conjugation to Ga ₂ -Tf	Wang et al., 2000
Toxin CRM107		K562, HL60	Dose-dependent inhibition	Hatano et al., 1993
		K562, HL60	Inhibition of both cells	Bérczi et al., 1993b
		K562	Enhanced cytotoxicity	Fritzer et al., 1992
		Patients	Enhanced cytotoxicity	Sizensky et al., 1992
	PHPMA	P388	Conjugates kill the leukemia cell	Faulk et al., 1990
	Liposome-PEG	C6 glioma	Polymer conjugated, Tf-targeted	Št'astný et al., 1999
			Encapsulated in Tf-liposome-PEG	Eavarone et al., 2000
	Thioether bond	scU87Mg, tumor-bearing mice	Effective in vitro but ineffective in vivo	Engebraaten et al., 2002
		Rat-bearing U251MG	Chloroquine block the toxicity	Hagihara et al., 2000
		Patients	Interstitial microinfusion	Laske et al., 1997
MMC	Glutaric anhydride spacer	K562 and SNB75	Cytosol translocation	Johnson et al., 1988
		HepG2 and cultured rat hepatocyte	Inhibited the growth of the HepG2 cell	Tanska et al., 2001
		HL60	TfR-mediated internalization	Tanska et al., 1998
Daunorubicin	Glutaraldehyde	Sarcoma 180		Tanska et al., 1996
Insulin	Disulfide	NCI-H69 (SCCL)	10 times effective over free drug	Bejaoui et al., 1991
		Caco-2 and diabetic rat	A slow but prolonged hypoglycemic effect and dose-dependent	Xia et al., 2000
Cisplatin	Tf-PEG-liposome	MKN45P, tumor bearing mice	Increase drug level in tumor cells and higher animal survival rates	Inuma et al., 2002
Chlorambucil	Maleimide spacer	MCF7, MOLT4	Ester or acid-sensitive link, inhibit growth of tumor cells	Beyer et al., 1998
F(ab') ₂	Maleimide spacer	K562	Acid-labile conjugates	Wellhöner et al., 1991
Ricin A	Disulfide	In vitro	Intracellular pathway	Kornfeld et al., 1991
α-IFN	Liposome-PLL	MBT2	Tf-PLL promotes delivery and enhances antiproliferative activity	Liao et al., 1998

α-IFN, α-interferon; F(ab')₂, anti-tetanus fragments; MMC, mitomycin C.

bicin and free drug in sensitive KB-3-1 and in multidrug-resistant KB cell lines (Fritzer et al., 1996). The conjugate was observed more effective with IC₅₀ concentrations of 0.006 and 0.028 μM compared with 0.03 and 0.12 μM for doxorubicin in the sensitive and resistant cells, respectively. For highly multidrug-resistant cells, the conjugate inhibited the cells with IC₅₀ of 0.025 to 0.2 μM, whereas doxorubicin did not exert any cytotoxicity even at concentration of 1 μM (Fritzer et al., 1996). These results demonstrated that Tf-doxorubicin conjugate is effective against multidrug-resistant tumor cells.

The mechanism by which the Tf-doxorubicin exerts its cytotoxicity has been studied intensively. Interaction of a Tf-doxorubicin conjugate with isolated transferrin receptors shows a similar binding affinity as that of Tf. The dissociation of the conjugate from the isolated TfR

occurred with time-dependent kinetics, similar to those of Tf when the experimental conditions mimicked the physiological steps of Tf recycling (Ruthner et al., 1994). The equilibrium binding and dissociation characteristics of Tf-doxorubicin at 0°C using K-562 cells were also compared with that of Tf. The results revealed that conjugation of doxorubicin to Tf does not affect qualitatively the iron-donating property of Tf. However, some difference was observed between the kinetic parameter characterizing the endocytosis and recycling of Tf and conjugate at 37°C (Bérczi et al., 1993a). The binding of Tf-doxorubicin on either isolated plasma membrane or viable tumor cells was also found to cause an inhibition of transplasma membrane electron transport and the NADH-ferricyanid reductase, which are essential for cell growth (Faulk et al., 1990; Sun et al., 1992). The cyto-

tosatic effects of doxorubicin are attributed to its ability to intercalate with DNA or to inhibit specific enzymes. The resistance toward doxorubicin treatment is thought due to overexpression of the MDR-1 gene resulting in the synthesis of P-glycoprotein, which functions as a pump and is capable of removing doxorubicin from the cytoplasm (Endicott and Ling, 1989). In contrast, the Tf-doxorubicin appears to exert its cytotoxicity through a transmembrane mechanism, instead of intercalating with DNA (Barabas et al., 1992; Bérczi et al., 1993a; Fritzer et al., 1996; Lai et al., 1998). Conjugates of doxorubicin with Tf thus offer a novel approach to target drugs directly to tumor cells. The specificity of this delivery system will likely overcome drug resistance and allow low doses of drug to be used, which will minimize or avoid the potentially lethal side effects experienced with conventional doxorubicin therapy.

2. *Transferrin-CRM107*. A conjugate of Tf and a point mutant of diphtheria toxin, Tf-CRM107, was shown to exhibit dose-dependent antitumor activity against solid human gliomas in nude mice (Laske et al., 1994). When Tf-CRM107 was intratumorally infused into patients with malignant brain tumors, over 60% of the patients exhibited tumor responses by a reduction in the tumor volume (Laske et al., 1997). However, patients receiving high doses of Tf-CRM107 developed toxicity indicative of small vessel thrombosis or petechial hemorrhage evidenced by magnetic resonance images. Similar toxicity was observed after intracerebral injection of Tf-CRM107 into rats at a total dose of 0.025 μg (Hagihara et al., 2000). The neurological deficit was attributed to the endothelial damage due to the low level of TfRs expressed by the capillary endothelial cells in the brain (Laske et al., 1997; Hagihara et al., 2000). To prevent this damage to the vasculature, chloroquine was systematically administered with Tf-CRM107 infused intracerebrally in rats (Hagihara et al., 2000). The results showed that the maximum tolerated dose of Tf-CRM107 was shifted from 0.2 to 0.3 μg . Chloroquine treatment completely blocked the brain damage detected by magnetic resonance images and caused by intracerebral infusion of 0.05 μg of Tf-CRM107. Furthermore, chloroquine treatment had little effect on the antitumor efficacy of Tf-CRM107 in nude mice bearing s.c. U251 gliomas (Hagihara et al., 2000). Therefore, the i.v. injection of chloroquine during intracerebral infusion of Tf-CRM107 may protect the vasculature, thus permitting less toxicity to the brain whereas allowing greater doses of Tf-CRM107 to be delivered to the tumor to further improve the response rate of this new cancer therapy.

3. *Others*. A variety of other therapeutic agents has also been attempted to be delivered into various cell lines by conjugated with Tf (Table 2). Chlorambucil (leukeran), another anticancer drug used clinically against chronic lymphatic leukemia, lymphomas, and advanced ovarian and breast carcinomas, is limited by its toxic side effects. The Tf-chlorambucil conjugate exhibited IC_{50} val-

ues ca. 3 to 18-fold lower than those of chlorambucil in the MCF7 mammary carcinoma and MOLT4 leukemia cell line. And preliminary toxicity studies in mice showed that this conjugate can be administered at higher doses compared with unbound chlorambucil (Beyer et al., 1998). Transferrin-mitomycin C (MMC), the chemotherapeutic DNA cross-linking agent, was also shown to be a useful hybrid as a receptor-mediated targeting system (Tanaka et al., 1996, 1998, 2001). The Tf-MMC conjugate bound and was internalized into various cells. The proliferation of the cells was inhibited by Tf-MMC in vitro.

A liposomal carrier system, which was produced by using small unilamellar liposomes made of pure phospholipids chemically cross-linked to human Tf, was reported to interact specifically with leukemia HL60 cells, and the conjugate was subsequently internalized by active receptor-mediated endocytosis (Sarti et al., 1996; Singh, 1999). Transferrin-coupled liposome, in which Tf was coupled to the distal ends of liposome polyethylene glycol (PEG), was shown to target specifically to C6 glioma in vitro. Doxorubicin encapsulated within Tf-coupled liposomes could enhance the uptake of free doxorubicin via the receptor-mediated mechanism (Eavarone et al., 2000). Liposome-entrapped α -interferon (α -IFN) when conjugated with Tf-polylysine (Tf-PLL) exhibited the antiproliferative effect against murine bladder tumor cell MBT2, and cell uptake of Tf-PLL-liposome was markedly enhanced in a dose-dependent manner. There was also a strong correlation between antiproliferative activity and uptake of liposome by the tumor cells, indicating that Tf-PLL-liposome promotes intracellular delivery of α -IFN and enhances the effect of α -IFN against MBT2 cell growth (Liao et al., 1998).

Transferrin-pendant type immunoliposome (TF-PEG-ILP) was shown to have a higher uptake to K-562 cells in vitro compared with nontargeted liposomes. The TF-PEG-ILP, examined in the B16 melanoma-bearing mice, exhibited a prolonged circulation time, a low liver uptake and concomitantly high accumulation into the tumor tissue and longer residence. Liposomes conjugated with anti-TfR have also been used for specific drug delivery. A liposome-immobilized anti-Tac (a monoclonal antibody against the IL-2 receptor) and anti-TfR (a monoclonal antibody against transferrin receptor) was compared for the specific binding, internalization, and intracellular drug delivery to adult T-cell leukemia (Hege et al., 1989). It was found that there was a better growth inhibition profile of anti-TfR-coupled liposome over anti-Tac-coupled liposomes bearing methothrexate- γ -aspartate, a liposome-dependent cytotoxic drug.

VII. Transferrin in Gene Delivery

The success of gene therapy relies on the ability of gene delivery systems to selectively deliver therapeutic genes to a sufficient number of target cells yielding expression levels that impact the disease state. Viral

vectors generally facilitate highly efficient transfer and expression of foreign genes, but attempts to modify their target cell specificity have proven difficult (Schnierle and Groner, 1996). Moreover, viral vectors can be immunogenic, cytopathic or recombinogenic; for example, adenoviral vectors can induce host immune response, thus rendering their repeated applications (Yang et al., 1994). Nonviral vectors including molecular conjugates and cationic liposomes are being exploited as promising alternatives. However, gene delivery employing these nonviral vectors suffers from low transfection efficiency. The receptor-mediated (such as TfR-mediated) gene delivery has an attractive feature since it provides an opportunity to achieve cell-specific delivery of DNA complexes; in the mean time it might also enhance the transfection efficiency. Therefore, this kind of molecular conjugate vector may have many potential applications in gene therapy.

A. Transferrin-Polylysine-DNA Conjugates

1. *General Methods of Preparation.* DNA is a large polyanionic molecule, which is not internalized per se by eukaryotic cells. Therefore, it has to be condensed to a size that allows it to be taken up into cells. In addition, the negative charges of the DNA have to be masked. Many different polycationic molecules have been used for this purpose, including polyornithine, histones, and

polyethyleneimine. However, the most widely used polycation is polylysine since it is available in a large variety of molecular weights, and it can also be easily degraded by cells (Duncan, 1992). The ϵ -amino group of lysine is positively charged at physiological pH and is a good target to covalently attach targeting ligands, e.g., Tf. Several coupling methods can be used (Baeumert and Fasold, 1989; Means and Feeney, 1990; Brinkley, 1992). Briefly, the ϵ -amino group of polylysine is modified with bifunctional linkers containing a reactive ester. Examples include *N*-succinimidyl-3-[2-pyridyldithio]propionate (SPDP), succinimidyl-4-[*p*-maleimidophenyl]butyrate, and *N*-(ϵ -maleimidocaproyloxy)succinimide. With the aid of these compounds, a thiol reactive group can be added to the polylysine molecule. Subsequent reactions with a thiol lead to production of a disulfide or thioether bond between polylysine and transferrin. The conjugates will then be purified by dialysis, chromatography, or preparative gel electrophoresis. The ligand-polylysine ratio can be characterized using acid urea gel electrophoresis (McKee et al., 1994), ninhydrin assay, and ligand (Tf) analysis.

2. *Uptake of DNA Particles.* Transferrin-polylysine conjugates have been shown to be efficient carriers for the introduction of genes into many cells and cell lines (Table 3). DNA complexed to Tf-polylysine is introduced into cells by receptor-mediated endocytosis, since the

TABLE 3
Therapeutic gene delivery using polycation-based vectors with attachment to a targeting Tf

Vectors	Biological Evaluation	Comments	References
Tf-PEI/DNA	A/J tumor-bearing mice Melanoma cell lines and mice Tumor bearing mice B16F10; Neuro2A; K562 cells	100- to 500-fold gene expression in tumor Similar transfection efficiencies as adenovirus in mice Efficient transfer gene in tumor and lung Reduced transfection	Kircheis et al., 2001 Wightman et al., 1999 Kircheis et al., 1999 Ogris et al., 1998
Polymer-PLL-DNA-Tf	Human K562 32 P-dCTP cells	15-fold increase in transfection and resistance to serum proteins Restricted to cytoplasmic	Dash et al., 2000 Fisher et al., 2000
Tf-PEI-PEGylated DNA	Tumor-bearing mice	Observation of reporter gene expression with low toxicity	Ogris et al., 1999
Tf-PLL/DNA	HeLa HeLa cells; WI-38; MRC-5; KB; CFT1 K562 Hematopoietic HD3 A549; BNL; CL.2; H225; NIH 3T3; Rat-1 HL-60	Schiff's base linker gave higher transfection than disulfide linker SA/ biotin linker enhance transfer Adenovirus enhance gene transfer Other polycations promoted transfection; chloroquine increases gene transfer; monensin blocks transfection Gene transfer occurred and enhanced by chloroquine Glycerol-enhanced transfection Inhibition of cell proliferation	Schoeman et al., 1995; Uike et al., 1998 Cotten et al., 1992; Curiel et al., 1991 Cotten et al., 1990; Wagner et al., 1990, 1991 Zenker et al., 1990 Zauner et al., 1996 Citro et al., 1992
Tf-PLL/DNA lipid	Myogenic cells	Serve as a potential skeletal muscle vector	Feero et al., 1997
Tf-PNA/DNA	Myogenic cells	PEI required to enhance transfection	Liang et al., 2000
Adenovirus-Tf-PLL/DNA	HeLa; BNL CL.2 cells Autologous rabbit jugular vein segments Canine hemophilia B; canine epithelial cells	100% transfection in both adenovirus or TfR-rich cells In vivo gene therapy is likely Over 50% transfection efficiencies	Wagner et al., 1992 Kupfer et al., 1994 Lozier et al., 1994

transfection was found to be enhanced by several factors, such as increasing the TfR density through treatment of the cells with the cell-permeable iron chelator desferrioxamine; and interfering with the synthesis of heme with succinyl acetone treatment, as well as stimulating the degradation of heme with cobalt chloride treatment (Cotten et al., 1990). The Tf-based gene transfer has been termed as "transferrinfection" (Wagner et al., 1990). The transferrinfection has been found to be influenced by the composition of DNA complexes (Wagner et al., 1991). Optimum Tf-PLL conjugates condensed DNA into a toroid of about 80 to 100 nm in diameter, as did PLL alone. Excess transferrin in PLL conjugate is detrimental to transfection (Wagner et al., 1991). In contrast, substituting some unconjugated PLL for Tf-PLL improved condensation of DNA and transfection. Ten to twenty Tf molecules per complex gave rise to an optimum transfection, but insufficient PLL or excess Tf failed to produce fully condensed DNA (Wagner et al., 1991). This study suggests that condensation of DNA is necessary for optimum transfection efficiency.

The transferrinfection was assessed with pRSV LuC plasmid on chicken erythroblastoid cells (Zenker et al., 1990). The results showed that Tf-PLL₍₂₇₀₎ gave rise to higher transfection efficiency than Tf-protamine and that most of the cells were shown to undergo transfection using β -galactosidase plasmid and analyzed by fluorescence activated cell sorter. Transferrinfection in these cells was found to be less than that obtained with conventional transfection protocols such as a DEAE-dextran. However, no cytotoxic effects were observed using the Tf-PLL method, in comparison, the DEAE-dextran killed 30 to 40% of the transfected cells. Either addition of chloroquine or repeated addition of the complex daily can improve the transferrinfection. Chloroquine at a concentration of 100 μ M was confirmed to augment luciferase expression in K-562 cells, while at 50 μ M or less failed to exert its effect (Cotten et al., 1990). Alternatively, adenovirus either administered to the transfection medium or coupled to the Tf-PLL/DNA was found to enhance the transferrinfection in a variety target cells (Curiel et al., 1991; Cotten et al., 1992).

The avidin/biotin technology has been applied in coupling the Tf and polylysine, and the efficiency of this system was evaluated using pRSV luciferase plasmid on HeLa cells (Schoeman et al., 1995). Optimum conditions for gene transfer was found to be 1.5:1 ratio of biotin to Tf, 1:1 ratio of avidin to Tf, 10:1 ratio of biotin to polylysine, and probably equal ratios of Tf to polylysine and DNA phosphate to lysine-free residues on polylysine. Some DNA transfection was observed in the absence of Tf. However, incorporation of Tf enhanced transfection by ca. 5-fold in the presence of chloroquine.

Tf-PLL-DNA conjugates provide a very efficient vector for gene transfer in some tissue culture cells. In K-562 cell line, virtually 100% of the cell population was found to express the transfected reporter gene for a protracted

period of days (Cotten et al., 1993). Such a delivery system was also shown to be efficient for the selective delivery of oncogene-targeted antisense oligodeoxynucleotides (Citro et al., 1992). It was observed that exposure of HL-60 cells to the *myb* antisense-Tf-polylysine complex resulted in rapid and profound inhibition of proliferation and loss of cell viability much more pronounced than that occurring in cells exposed to free *myb* antisense oligodeoxynucleotides, although the Tf-polylysine-*myb* sense complex or the Tf-polylysine conjugate alone had no effect on HL-60 cell proliferation and viability.

Furthermore, this system may also allow great size and sequence variety of DNA to be delivered into cells. The commonly used recombinant adenovirus vectors allow delivery of 8 kb of additional sequences. The delivery efficiency of a Rous sarcoma virus-luciferase gene present on either an 8-kb circular DNA plasmid or on a 48-kb circular DNA cosmid was assessed using HeLa cells (Cotten et al., 1992). It was noted that very little delivery of a 48-kb circular DNA using the Tf-PLL system in the absence of adenovirus and in the presence of chloroquine whereas the small plasmid was found to be delivered successfully into the cells. However, in the presence of adenovirus, the resulting luciferase activity is virtually the same for both the 8- and 48-kb DNA molecules testing DNA quantity from 6 to 0.5 μ g, which suggests that large DNA is delivered into the cell. However, it is not clear whether fragmentation of the large molecule occurs.

3. Problems Associated with Transferrin-Polylysine-Based Gene Delivery. Binding to serum proteins to polyelectrolyte gene delivery complexes is thought to be an important factor limiting bloodstream circulation and restricting access to targeted tissues. Moreover, protein binding can also inhibit transfection activity in vitro. DNA delivered by receptor-mediated endocytosis also suffers from the limitation that endocytosed DNA is trapped in intracellular vesicles and later largely destroyed by lysosomal action. Different strategies have been developed to stabilize polyelectrolyte gene delivery vectors and to ensure the release of DNA from internal vesicles.

It has been shown that PEGylated conjugates of Tf-PLL-DNA strongly reduced plasma protein binding as a result of reduced surface charges (Ogris et al., 1999). Administration of the PEGylated complexes through the tail vein results in reporter gene expression in some mice (Ogris et al., 1999). Recently, a novel polymer-coated pLL-DNA complex was produced from poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA), which bears pendent oligopeptide (Gly-Phe-Leu-Gly) side chains and reacts with PLL. It was found that the resulting nanoparticulate vectors completely resist attack by serum proteins (Dash et al., 2000; Fisher et al., 2000). The attachment of Tf to the complex significantly enhanced both cell uptake (6-fold) and transfection activity (15-

fold) compared with either uncoated pLL-DNA or untargeted pHPMA-coated complexes (Dash et al., 2000). Moreover, the idea can be used to design new drugs by incorporating different oligopeptide sequences to regulate the degree of activation of complexes as required. The attachment of targeting ligands onto the surface of gene delivery vectors via unreacted ester groups is both novel and extremely flexible. Accordingly, this class of "polymer-modified DNA prodrugs" is a promising candidate for targeted gene delivery both in vitro and potentially in vivo (Dash et al., 2000).

Different strategies have been developed to ensure the release of DNA from internal vesicles. The addition of lysomotropic agent chloroquine to the transfection medium results in increased gene expression in a variety of cell lines (Cotten et al., 1990; Zenker et al., 1990). This is attributed to accumulation of chloroquine in acidic vesicle and results in raising the pH of these vesicles, thus inhibiting lysosomal enzymes that degrade DNA (Zauner et al., 1996). The high amount of chloroquine that accumulates in the acidic vesicle also destabilizes the endosome. However, chloroquine displays some toxicity toward cells. Application of glycerol has also been demonstrated to enhance gene transfer (Zauner et al., 1996), probably by weakening of the endosomal membrane, thus allowing polylysine to disrupt the membrane. Incorporation of viruses may be another alternative to enhance the release of therapeutics from internal vesicles. The addition of adenovirus to Tf-PLL-DNA complexes augmented transfection levels in a dose-dependent manner (Curiel et al., 1991; Cotten et al., 1992). Adenovirus may also be directly coupled into the DNA complex via a biotin-streptavidin bridge. Such an approach allows the colocalization of both the DNA particle and the adenovirus in the same endosome, therefore facilitating the endosome disruption and allowing higher gene transfection efficiency in a broad variety of cells and cell lines (Wagner et al., 1992; Kupfer et al., 1994; Lozier et al., 1994). However, the drawback of using adenovirus to enhance cytoplasmic delivery may be the inflammatory response of cells, as well as their immunogenicity in vivo. Therefore, other strategies need to be sought to enhance the efficiency of gene transfection.

B. Transferrin-Lipoplexes

Cationic liposomes are composed of positively charged lipid bilayers that can be complexed to negatively charged naked DNA by simple mixing. The resulting cationic liposomes-DNA complexes (lipoplexes) formed by a combination of electrostatic attraction and hydrophobic interaction have been used extensively as nonviral vectors for the intracellular delivery of reporter or therapeutic genes in culture and in vivo (Lasic and Templeton, 1996). The majority of lipoplexes is thought to take up via endocytosis, followed by their release from an early endosomal compartment. These cationic

liposome-DNA complexes suffer from low gene transfer efficiency, large particle size, poor stability and more importantly, lack of targeting capability (Pirollo et al., 2000). In addition, application of lipoplexes in vivo is also limited by the inhibition of serum. However, this can be dramatically improved when the liposomes bear a ligand recognized by a cell surface receptor, which will facilitate the entry of DNA into cells through initially binding of ligand by its receptor on cell surface followed by internalization of bound complex. Sufficient DNA will then escape the endocytic pathway to be expressed in the cell nucleus. Transferrin has demonstrated its ability to direct cationic liposomes to receptor-bearing cells (Cheng, 1996; Simões et al., 1999). Association of Tf with lipoplexes, in particular, the negatively charged ternary complexes, significantly overcame the inhibitory effect of serum and facilitated efficient transfection in many cell lines, including HeLa, K-562 cells, and lung carcinoma cells Calu3 and H292 cells (Simões et al., 1998; de Ilarduya and Düzgünes, 2000; Yanagihara et al., 2000; Kono et al., 2001). This vector was also effective in transfection in epithelial and lymphoid cell lines, as well as human macrophages, especially with the use of optimized lipid/DNA ($\pm$) charge ratios (Lima et al., 1999).

Considerable research has been made toward delivery of the tumor suppressor gene p53 via cationic liposome-based vectors (Xu et al., 1997, 1999, 2001, 2002; Seki et al., 2002). The p53 gene has been shown to be involved in the control of DNA damage-induced apoptosis, and malfunction of this p53-mediated apoptotic pathway could be one mechanism by which tumors become resistant to chemotherapy or radiation. Tf-lipoplex has demonstrated high efficiency in tumor-targeted gene delivery and long-term therapeutic accuracy in systemic p53 gene therapy for both human head and neck cancer (Xu et al., 1999) and prostate cancer (Seki et al., 2002). It has been shown that Tf significantly increased the transfection efficiency for JSQ-3 cells, established from a squamous cell carcinoma of the head and neck, in culture (6- to 10-fold increase) when compared with the liposome alone even in the presence of high levels of serum (Xu et al., 1997, 2001). The intratumoral delivery of p53 gene to mouse tumor xenograft model of human prostate PC-3 carcinoma cells using a Tf-lipoplex vector resulted in inhibition of tumor growth and an increase in animal survival (Seki et al., 2002). Injection of Tf-liposome-p53 via the tail vein to nude mice bearing DU-145 subcutaneous tumors resulted in a high level of exogenous wild-type p53 expression. In contrast, no significant exogenous p53 expression was observed in tumors from the mouse injected with nontargeted liposome-p53 (Xu et al., 2002). The in vivo efficacy of Tf-lipoplex-mediated p53 gene therapy was further investigated and resulted in improved efficacy in systemic p53 gene therapy of human prostate cancer. Moreover, when combined with radiation, the Tf-lipoplex-p53-treated group exhibited complete tumor regression and showed no signs of re-

currence 6 months after treatment (Xu et al., 2002). The long-term efficacy of Tf-liposome-p53 radiosensitization was also observed in a head and neck cancer animal model (Xu et al., 1999). Therefore, a novel strategy combining current molecular medicine with conventional chemotherapy and radiotherapy has the potential in the clinical treatment of cancer.

A recent study has elucidated the structure and formation process of this efficient and efficacious Tf-lipoplex (Xu et al., 2002). The optimal formulation for prostate cancer cells DU-145 was found to be a DNA:lipid:Tf ratio of 1 μ g/10 nmol/12.5 μ g, similar to that optimized for the human head and neck cancer model (Xu et al., 1999). The transfection efficiency for DU-145 cells using this formulation is comparable to that of the head and neck cancer cell line JSQ-3, with an in vitro and in vivo efficiency of 60 to 70% and 20 to 30%, respectively (Xu et al., 1999, 2002). The optimal Tf-lipoplex particle size on gene transfection efficiency was found to be 50 to 90 nm. The particle has a highly compact structure, and resembles a virus particle both in architecture and its uniformly small size (Xu et al., 2002). This virus-like compact nanostructure is likely to be the key to their high gene transfer efficiency and efficacy both in vitro and in vivo. A model of self-assembly process of this nanoparticles was proposed. It may involve attachment of DNA strand to Tf-liposome via electrostatic interaction; formation of individual lamellar structures along the DNA strand; and finally formation of a condensed lamellar core structure encapsulated by a Tf-coated membrane. This self-assembly process and transferrin-facilitated DNA co-condensation model contributed to the small size and effectiveness of the Tf-lipoplex nanostructure (Xu et al., 2002). These studies suggest that the systemic delivery of normal tumor suppressor gene p53 by a non-viral tumor-targeted delivery system as a new therapeutic intervention has the potential to critically impact the clinical management of cancer.

C. Other Vectors Using Transferrin as a Targeting Ligand

Each gene transfection method currently prevailing has inherent drawbacks. Therefore, new vectors with higher transfection and lower toxicity need to be invented to achieve effective gene therapies in vivo. Recently, a new vector, which conjugates the naked DNA with Tf via avidin/biotin technology, has been developed and has shown potential in gene delivery in vivo (Sato et al., 2000). The transgenes including β -galactosidase gene, green fluorescent protein (GFP) gene, and herpes simplex virus thymidine kinase (HSV-TK) were conjugated to Tf by a biotin-streptavidin bridging. Briefly, biotinylated Tf was applied to TfR-immobilized column, streptavidin dissolved in phosphate-buffered saline was applied, followed by addition of biotinylated plasmid and incubated for a certain period, the resulting conjugates were eluted from the column, and the apo-DNA conju-

gate was saturated with iron and dialysis (Sato et al., 2000). The conjugates constructed in such a way are stoichiometrically controllable. Transfection of Tf- β -galactosidase plasmid conjugate to human erythroleukemia cells K-562 via TfR was achieved without the aid of any lysosomotropic agents. The transfection efficiency was superior to those of lipofection (1% staining) and retroviral vector (5%) and slightly lower than that of adenovirus (70%). Similarly, the high level of expression was also confirmed in other tumor cells such as M7609 and TMK-1. In contrast, in normal diploid cells (HEL), gene expression was negligible owing to a low level of TfR. It was also noticed that administration of GFP gene conjugates systemically through the tail vein to nude tumor-bearing mice caused expression of GFP mRNA almost exclusively in tumors and to a much lesser extent in muscles (Sato et al., 2000). To exploit the therapeutic applicability of this kind of vector, suicide gene therapy using Tf-HSV-TK gene conjugate for massively metastasized K-562 tumors in severe combined immunodeficient mice was conducted, and the results showed a marked prolongation of survival and significant reduction of tumor burden (Sato et al., 2000). This novel vector has no size limitation for the gene to be conjugated and no apparent side effects in vivo. Furthermore, it is noncytotoxic and has relatively high transduction efficiency. Therefore, it is potentially applicable for efficient gene transduction in vivo as well as in vitro gene delivery to tumor tissue.

A novel system based on the use of nanoparticles formed by coacervation of chitosan or gelatin and plasmid DNA has also been developed. Cross-linked gelatin coacervates incorporated 25 to 30% (w/w) DNA, with a size range of 200 to 700 nm, provided some protection against degradation by nuclease in serum (Truong-Le et al., 1998). By conjugation with transferrin onto the nanosphere, the transfection of culture cells with a luciferase plasmid was enhanced by two orders of magnitude, and coencapsulating the endolysolytic agent chloroquine enhanced transfection ability by a further order of magnitude (Leong et al., 1998; Truong-Le et al., 1998). The size of optimized chitosan-DNA nanoparticles is in the range of 100 to 250 nm, with 35.6% DNA encapsulated in the particulates. These coacervates had a transfection activity similar to ligand-targeted gelatin nanoparticles with chloroquine, but transfection of coacervates was not improved either by Tf or by chloroquine (Mao et al., 2001).

VIII. Transferrin and Transferrin Receptor in Drug and Gene Delivery across the Blood-Brain Barrier

Many therapeutic molecules such as anticancer drugs, proteins, and peptides are generally excluded from transport from blood to brain, owing to the negligible permeability of these drugs to the brain capillary endo-

thelial wall, which makes up the BBB in vivo. However, therapeutics may be delivered to the brain with the use of strategy of coupling therapeutics to a BBB drug transport vector. Brain capillary endothelia cells possess specific receptor-mediated transport mechanisms that potentially can be exploited as a means to delivery therapeutic molecules to the brain.

A. OX26 As an Efficient Brain Drug Transport Vehicle

Transferrin itself is limited as a brain drug transport vector since the transferrin receptors are almost saturated under physiologic conditions due to high endogenous plasma concentrations of transferrin (Seligman, 1983). However, the antibodies that bind to the TfR have been shown to selectively target BBB endothelium due to the high levels of TfR expressed by these cells (Friden, 1994; Bickel et al., 2001; Moos and Morgan, 2001). Therefore, these antibodies are potential carriers for the delivery of therapeutic agents to the central nervous systems. With a mouse monoclonal antibody against the rat TfR, OX26, a high abundance of transferrin receptors at the brain microvascular endothelium has been detected (Jefferies et al., 1984). The OX26 monoclonal antibody against the rat TfR is likely to be a candidate in the delivery of therapeutic agents to the brain.

The OX26 binds to an extracellular domain on the TfR, distinct from the transferrin binding site, and does not interfere with Tf binding (Jefferies et al., 1984). A study on the binding and internalization of ^3H -OX26 using isolated bovine brain capillaries demonstrated that, similar to Tf, the OX26 was bound and internalized into the capillaries at either 37°C or 4°C (Pardridge et al., 1991). About 50% of the bound antibody was taken up into the capillary cytoplasm via endocytosis during a 2-h incubation period. The OX26 is also taken up by brain in vivo as deduced from an internal carotid artery perfusion/capillary depletion experiment in rats. The analysis of the postvascular supernatant showed that more than 65% OX26 had passed across the BBB into the brain extracellular space during the perfusion and the apparent volume of distribution in postvascular supernatant was higher for OX26 than for both cationized bovine serum albumin and IgG, indicating that receptor-mediated transcytosis appeared to be more efficient compared with absorptive-mediated transcytosis. The experiment of organ uptake of ^3H -OX26 following intravenous bolus injection showed the OX26 accumulated steadily in brain up to 5 h compared with a control monoclonal antibody of the same IgG 2A subclass (Pardridge et al., 1991). Furthermore, the serum radioactivity of ^3H -OX26 showed a rapid clearance after intravenous bolus injection (Pardridge et al., 1991). Therefore, the OX26 appears to be the most efficient vector candidate in brain drug delivery (Wu et al., 2002).

B. Preparation of OX26 Drug Conjugates

It is necessary to devise appropriate conjugation strategies whereby the nontransportable therapeutics are linked to the vector in such a way that bifunctionality of the conjugates are retained. That is, the conjugates must retain a high affinity to both the BBB vector and the cognate receptor that is attached to the delivery system. Several approaches have been used in linking drugs to transport vectors, including the combined use of organic chemistry, avidin/biotin technology, PEGylation technology, and genetic engineering technology.

Similarly as in the generation of Tf-drug conjugates, the chemical-based linkers employ activating reagents such as *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS), 2-iminothiolane (Traut's reagent), *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), which activate primary amino groups on surface lysine residues of either drugs or the transport vectors (Pardridge, 1999). This results in the formation of either a stable thioether linkage, which is comprised of only a single sulfur atom, or disulfide bonds in the case of using SPDP. A noncleavable linkage such as an amide bond may also be used in coupling the drug with the transport vector (Pardridge, 1999). Furthermore, the PEGylation technology is also applied in the conjugation of drug with the OX26 (Huwylar et al., 1996; Shi and Pardridge, 2000). In this case, a much longer spacer arm is comprised of a PEG moiety with a molecular weight of 2000 to 3400, which resolved the steric hindrance between the drug and delivery system.

The conjugation of drugs to BBB transport vectors is facilitated with the use of avidin/biotin technology. Avidin, an egg white protein, is a 64-kDa homotetramer that has four biotin binding sites per molecule (Green, 1990). The avidin/biotin bond is not covalent in nature, but binding is extremely strong with a dissociation constant (K_d) in the order of 10^{-15} M and dissociation half-life of ca. 89 days (Green, 1990). Although the avidin/biotin bond is stable in the circulation, it is labile at tissue depot sites (Pardridge, 1999). Therefore, the avidin/biotin system is ideal for drug delivery to tissues. Figure 6 shows the conjugation of a BBB vector OX26 with drug using avidin/biotin technology. The use of avidin/biotin technology applied to brain drug delivery involves the parallel mono-biotinylation of the drug, and the construction of a conjugate of the OX26 and an avidin analog, either avidin, or neutral forms of avidin such as streptavidin (SA) or neutral light avidin (Kang et al., 1995). Such a conjugate may either be prepared chemically using stable thio-ether linkages or may be genetically engineered from vector/avidin fusion gene and the expression of fusion proteins (Li et al., 1999; Penichet et al., 1999). It is worth mentioning that the drug must be mono-biotinylated. Because of the multivalency of avidin binding of biotin, multibiotinylation of the drug would cause the formation of high-molecular

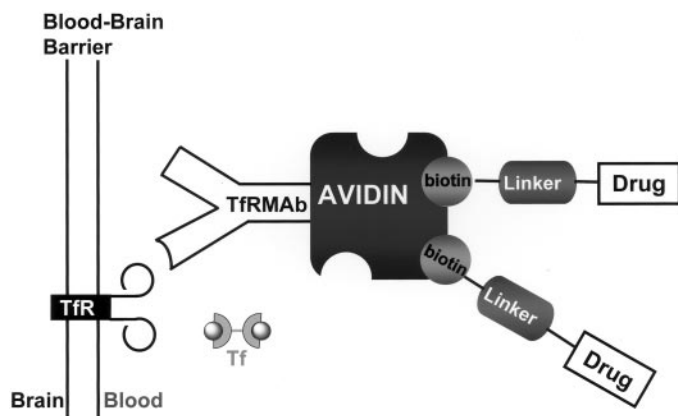


FIG 6. A general scheme of a covalent conjugate of a monoclonal antibody (TfR/mAb) to the TfR using the avidin/biotin technology. The linker between the drugs and biotin can be either a disulfide or noncleavable amide bond (Bickel et al., 1993).

mass aggregates, which would be rapidly cleared by the reticulo-endothelia system in vivo (Pardridge et al., 1998).

This biotechnology has been used in the conjugation of OX26 with the brain-derived neurotrophic factor (BDNF) (Pardridge et al., 1998; Zhang et al., 2001; Zhang and Pardridge, 2001a,b) and a neuropeptide, vasoactive intestinal peptide analog (VIPa) (Bickel et al., 1993). Briefly, a 1:1 conjugate of the OX26 mAb and SA was prepared via a stable thiol-ether linkage using OX26 thiolated with Traut's reagent and SA activated with MBS. BDNF-PEG 2000-biotin was prepared with ^{125}I -BDNF as an internal standard, and the BDNF was either biotinylated at the ϵ -amino group of surface lysine residues or at carboxyl moieties on surface glutamate or aspartate residues. The BDNF-PEG 2000-biotin/SA-OX26 was then formed by mixing 2 mg of PEG 2000-biotin and 8 mg of OX26/SA followed by purification of the conjugate from aggregates or unconjugated BDNF using a Sephacryl S300 HR column. The final yield of conjugate was 4.5 mg, and 14% of this was BDNF and 86% was OX26/SA, which means a 7:1 ratio of BDNF and the OX26/SA.

Another class of linker strategies is based on genetic engineering. In this approach, a fusion gene is prepared in which the cDNA for a recombinant protein is fused to the cDNA encoding for the transport vector, followed by preparation of fusion gene and expression of the fusion protein (Li et al., 1999). It is important that recombinant proteins will not be biologically active following fusion to a transport vector. This approach can be used with a combination of the avidin/biotin technology. It involves initial construction of a fusion gene encoding the cDNA for avidin and cDNA for the transport vector. Such an avidin mAb fusion gene has been produced and fusion protein has been expressed, which retained the bifunctional characteristics of antigen binding and biotin binding (Shin et al., 1997; Li et al., 1999).

Recently, a novel vector based on immunoliposomes (antibody-directed liposomes) showed potential both for brain drug and gene delivery (Huwylar et al., 1996; Shi and Pardridge, 2000). The vector brings together liposome technology, pegylation technology, BBB-targeting technology, and plasmid-based therapeutic gene technology. Small molecule drugs or an exogenous plasmid DNA were incorporated into the interior of the neutral liposomes (Fig. 7). A PEG of 2000 Da molecular mass (designated PEG 2000) was attached to the liposome surface. A thiolated antibody, the OX26 murine mAb to the rat transferrin receptor, was coupled to the terminal end of the PEG 2000. The advantage to using PEG-conjugated immunoliposomes is that there is an increase in the drug-carrying capacity of the monoclonal antibody by up to 4 logarithmic orders in magnitude, which allows micromolar concentrations to be achieved in the brain resulting in many pharmacologically active small molecules (Shi and Pardridge, 2000).

C. Delivery of Therapeutics to the Brain

A wide range of therapeutics including small molecule drugs, proteins or peptides, and genes have been conjugated with OX26, and their efficacy in vivo or in vitro has been extensively studied (Table 2). Neurotrophins such as BDNF are potential neuroprotective agents that could be used in the treatment of a variety of neurodegenerative disorders (Table 4). However, BDNF, like other neurotrophin or protein-based therapeutics, does not undergo significant transport through the brain capillary endothelial wall, which makes up the BBB in vivo. Conjugates of BDNF to the OX26 could undergo receptor-mediated transcytosis through the BBB, which allows the efficacy of BDNF to improve. When a conjugate of BDNF and OX26 via PEG 2000 and avidin/biotin was administered intravenously daily to rats for 1 week after a 12-min period of transient forebrain ischemia, the neuronal density in the CA1 sector of the hippocampus was found to decrease $68 \pm 10\%$ 1 week after the ischemia (Wu and Pardridge, 1999). No neuroprotective effect was observed either for the unconjugated BDNF or unconjugated OX26. However, the hippocampal CA1

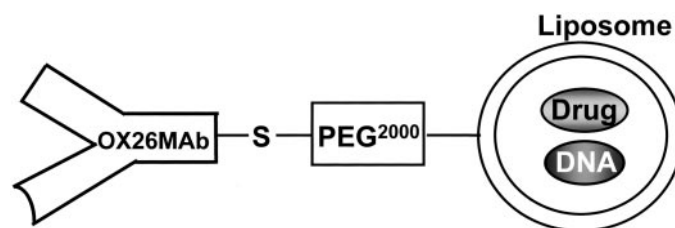


FIG 7. A brain drug vector based on immunoliposomes. Small molecule drugs or therapeutic genes are encapsulated in the interior of liposomes. Approximately 3000 strands of PEG of 2000 Da molecular mass (PEG 2000) are attached to the surface of liposomes to sterically stabilize the liposomes. The thiolated mAb OX26 is conjugated to the tail of ca. 1% the PEG strands. This vector is effective in the delivery of small molecule drugs or genes into the cells (Huwylar et al., 1996; Shi and Pardridge, 2000).

TABLE 4
Delivery of therapeutics to the brain by coupling with the OX26 vector

Therapeutics	Method of Preparation	Biological Evaluation	Special Remarks	References
VIPa	Avidin/biotin	Rat brain	Effective by 65% increase in cerebral blood flow	Bickel et al., 1993
PNA	SA/biotin	Rat brain	28-fold increased uptake after coupling the OX26-SA	Pardridge et al., 1995
Daunomycin	PEG-liposome	Rat brain	Expanding the loading by using PEG-liposome	Huwyler et al., 1996
BDNF	PEG-SA/biotin	3T3-TrB cell; rat brain	2-fold increased uptake compared with small molecule; neuroprotective effect in brain	Pardridge et al., 1998; Wu and Pardridge, 1999
		Rat brain (MCAO)	Reduce in cortical stroke volume	Zhang and Pardridge, 2001a,b
Antisense ODNs, PNA	SA/biotin	Alzheimer and HIV model; Rat brain	Increased cellular uptake ODNs in vitro; plasma protein inhibited the transport in vivo	Boado et al., 1998
Genes	PEG-liposome	Rat brain	Wide spread gene expression in the central nervous system	Shi and Pardridge, 2000
Antisense 16-mer PNA	Avidin/biotin	Rat brain with tumors	Gene expression in the brain in vivo can be used in antisense imaging	Shi et al., 2000
NGF	Chemically linked	Rat brain	Prevent degeneration of central NGF-responsive neurons	Kordower et al., 1994

neuronal density was normalized by i.v. administration of the BDNF-OX26 conjugate (Wu and Pardridge, 1999). Similarly, an increased brain uptake of the BDNF-OX26 conjugate was also noticed to a level of $0.144 \pm 0.004\%$ injected dose per gram of brain and a BBB permeability-surface area product of $2.0 \pm 0.2 \mu\text{l}/\text{min}/\text{g}$ (Pardridge et al., 1998). These studies suggest that BDNF may have neuroprotective effects on the brain if the neurotrophin is reformulated to minimize rapid systemic clearance of the peptide and allow for vector-mediated drug delivery through the BBB. Moreover, recent studies also show the potential of BDNF in the treatment of acute stroke if coupled with a brain drug transporter. In these studies, rats were subjected to 1 h of the middle cerebral artery in nitrous oxide, then ventilated with normal blood sugar, then the brain was reperfused. It has been observed that unconjugated BDNF failed to exert neuroprotection. In contrast, there was a 68 and 70% reduction in cortical stroke volume at 24 h and 7 days, respectively, after intravenous administration of $50 \mu\text{g}/\text{rat}$ of the BDNF conjugate. The marked neuroprotection in focal, transient brain ischemia is long-lasting and persists for at least 7 days after a 1-h middle cerebral artery occlusion (Zhang and Pardridge, 2001b). When adult rats subjected to 24 h of permanent middle cerebral artery occlusion (MCAO) was treated intravenously with the BDNF-OX26 conjugate at a dose of 1, 5, and $50 \mu\text{g}/\text{rat}$, the infarct volume was found to decrease by 6, 43, and 65%, respectively (Zhang and Pardridge, 2001a). Significant reduction in stroke volume was still observable even when the administration of the BDNF conjugate was delayed for 1 to 2 h after MCAO, although the pharmacological effects was progressively diminished in proportion to the time delay between MCAO and treatment (Zhang and Pardridge, 2001a). Apart from BDNF, other therapeutics such as polyamide nucleic acids

(PNAs), VIPa, and nerve growth factor have demonstrated the ability to cross the BBB and exert their pharmacological activities if coupling with the brain drug transporter (OX26) (Table 2). Furthermore, successful delivery of small molecule drugs, such as the antineoplastic agent daunomycin, to the rat brain, and widespread gene expression in brain after noninvasive i.v. administration of a 6- to 7-kb expression plasmid, encoding either β -galactosidase or luciferase, has been achieved by using a novel vector based on immunoliposomes (antibody-directed liposomes) (Huwyler et al., 1996, 2002; Shi and Pardridge, 2000).

Antisense oligonucleotides (ODNs) and PNAs are potential therapeutics for eradication of malignancies, viral infections, and antisense imaging, should these molecules be made transportable through the BBB in vivo. A cellular delivery system based on conjugates of the OX26 can be employed as a universal carrier for the transport of these peptides. It was shown that the cellular uptake and efficacy of 3'-biotinylation of phosphodiester (PO)-ODN was markedly increased in models of Alzheimer's disease and human immunodeficiency virus-acquired immunodeficiency syndrome (Boado et al., 1998). However, in vivo brain delivery studies demonstrated that 3'-protected PO-ODNs and PO-phosphorothioate (PS)-ODNs are subjected to endonuclease degradation, whereas PS-ODNs, protected at 3'-terminus by biotinylation, are resistant to exo/endonuclease degradation (Boado et al., 1998). Low efficiency in the transport of PS-ODNs across the BBB by the OX26 delivery vector was noticed as a result of the strong binding of these oligomers to plasma proteins. However, replacement of the deoxyribose/phosphate linkage by a polyamide-produced PNAs, a new generation of antisense molecules that are resistant to exo/endonuclease and protease degradation. These molecules, biotinylated at

the terminal group, could be transported into the brain by the OX26-SA delivery system, and the levels of brain uptake were found to be comparable to that of morphine (Boado et al., 1998).

Antisense radiopharmaceuticals could also be used to image gene expression in the brain in vivo. The imaging of structures within the brain with antisense radiopharmaceuticals requires that two conditions be met. First, radiopharmaceuticals must be able to traverse the BBB and brain cell membrane so that the radiopharmaceuticals can access the target. Second, the region of interest must overexpress the target mRNA. In a recent study, an antisense imaging agent was produced using an iodinated PNA, which hybridizes to the region around the methionine initiation codon of the luciferase mRNA, conjugated to the OX26 by using avidin/biotin technology (Shi et al., 2000). The ^{125}I -PNA conjugate was injected intravenously in anesthetized animals bearing brain tumors and killed 2 h later for frozen section of brain and film autoradiography. The results showed that tumors were imaged in all rats administered the ^{125}I -PNA that was antisense to the luciferase sequences and was conjugated to the OX26. In contrast no image of the luciferase gene expression was observed after administration of either the unconjugated antiluciferase PNA or a conjugated PNA that was antisense to the mRNA of a viral transcript (Shi et al., 2000). Therefore, all this evidence suggests that this kind of antisense molecule has great potential in the diagnosis and treatment of central nervous system disorders.

IX. Summary

In the past few decades, intensive studies have been made toward understanding Tf/TfR-mediated cellular iron uptake pathway. The identification of HFE as a hereditary hemochromatosis, DMT1 and ferroportin1 as iron transporters, and TfR2 as the second transferrin receptor represents a major breakthrough and provides insight into the mechanism of iron absorption, transport, and the cellular regulation of iron metabolism.

The strategy of exploiting Tf/TfR as a drug carrier system is based on elevated levels of transferrin receptors present on the surface of tumor cells. Therapeutic drugs including small molecule anticancer drugs, peptides, or proteins can be either chemically conjugated to Tf or encapsulated in the liposome. The resulting conjugates then undergo receptor-mediated endocytosis and subsequently release the drugs. Some of the expected advantages of Tf-drug conjugates are a preferable tissue distribution, prolonged half-life of the drug in the plasma, and controlled drug release from the conjugates. The stoichiometry and stability of the conjugate can be finely tuned by using different linkage strategies. Alternatively, the sequence of therapeutic peptides or proteins can also be incorporated into the structure of Tf through protein engineering. Such an approach offers

potential not only in targeted drug delivery but also for developing new therapeutic agents in the future.

Transferrin receptor has also been used as a general targeting molecule to direct DNA, condensed by a polycation such as polylysine, or encapsulated in the liposome, to rapidly dividing cells. The conjugated DNA is introduced into cells by a receptor-mediated pathway. The size and composition of the conjugates may influence the Tf efficiency. This kind of vector is very efficient for gene transfer in some tissue culture cells; when using a cationic liposome-Tf based vector, a tumor suppressor gene, such as p53 has been successfully delivered into tumors in vivo. However, generally speaking, it suffers from a lower Tf efficiency compared with viral vectors, as well as problems arising from interactions with serum proteins, which limit blood stream circulation and restrict access to the target tissues. Future work needs to focus on how to enhance the transfection efficiency. These may include chemically modifying the system, such as optimizing parameters affecting surface binding and associations and developing a specific mechanism to effectively release therapeutic genes from the endosome into the cytosol.

Delivery of drugs to the brain has been particularly challenging because of the BBB that restricts the passage of most therapeutics into the brain. Therefore, active targeting of the brain is crucial for effective treatment of central nervous disorders. The anti-TfR antibody, such as OX26, when coupled with therapeutics has been shown potential in the brain drug and gene delivery. In particular, the vector based on immunoliposome (antibody-directed liposome), which bring together liposome technology, pegylation technology, BBB-targeting technology, and plasmid-based therapeutic gene technology may have many applications for the diagnosis and therapy of a broad range of central nervous system disorders in the future.

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