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Structure and Dynamics of Drug Carriers and Their Interaction with Cellular Receptors: Focus on Serum Transferrin[#]

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Abstract

Highly proliferative cells have a dramatically increased need for iron which results in the expression of an increased number of transferrin receptors (TFR). This insight makes the transferrin receptor on these cells an excellent candidate for targeted therapeutics. In this regard, it is critical to understand at a molecular level exactly how the TFR interacts with its ligand, hTF. Understanding of the hTF/TFR pathway could, in theory, maximize the use of this system for development of more effective small molecules or toxin-conjugates to specifically target cancer cells. Many strategies have been attempted with the objective of utilizing the hTF/TFR system to deliver drugs; these include conjugation of a toxin or drug to hTF or direct targeting of the TFR by antibodies. To date, in spite of all of the effort, there is a conspicuous absence of any successful candidate drugs reaching the clinic. We suggest that a lack of quantitative data to determine the basic biochemical properties of the drug carrier and the effects of drug-conjugation on the hTF-TFR interaction may have contributed to the failure to realize the full potential of this system. This review provides some guidelines for developing a more quantitative approach for evaluation of current and future hTF-drug conjugates.

Keywords

transferrin; transferrin receptor; Trojan horse; chemotherapy

1. Introduction

Given the potential for detrimental effects due to the redox properties of the Fe²⁺/Fe³⁺ pair, once it enters the body iron that is acquired from the diet is tightly regulated. Specifically, iron, in the form of Fe³⁺, is carefully shielded from exposure to the aqueous environment of the blood by sequestration within each cleft of the iron binding and transport protein, human serum transferrin (hTF). An ~80 kDa bilobal (N- and C-lobes) glycoprotein secreted from the liver into the blood, hTF binds Fe³⁺ very tightly ($K_d \sim 10^{-22}$ M) [1], yet reversibly. Since hTF can bind iron in either or both lobes, four different hTF species (differing only in iron

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content) circulate in the blood: diferric (Fe_2hTF), monoferric N-lobe hTF ($\text{Fe}_\text{N}\text{hTF}$), monoferric C-lobe hTF ($\text{Fe}_\text{C}\text{hTF}$) and iron-free hTF (apohTF). At the pH of the serum (~7.4), Fe_2hTF binds with the highest affinity ($K_d \sim 4 \text{ nM}$) to the specific transferrin receptor (TFR) located on the cell surface of all actively dividing cells [2]. The two monoferric hTFs ($\text{Fe}_\text{N}\text{hTF}$ and $\text{Fe}_\text{C}\text{hTF}$), also form a stable high affinity complex with the TFR ($K_d \sim 36 \text{ nM}$ and $K_d \sim 32 \text{ nM}$, respectively) [2]. In contrast, at neutral pH apohTF binds very weakly, if at all. After clathrin-mediated endocytosis of the hTF/TFR complex [3], the lower pH within the endosome (~5.6), in conjunction with salt and an, as of yet, unidentified chelator, stimulates the release of iron from hTF with the active participation of the TFR. Critically, apohTF binds with high affinity to the TFR at endosomal pH, allowing the apohTF/TFR complex to return to the cell surface instead of being targeted to late endosomes or to the lysosome for degradation [4]. Either through dissociation from the TFR or displacement by Fe_2hTF or the monoferric hTF species [5], apohTF is released into the serum at the cell surface, where it is free to bind more Fe^{3+} and repeat the cycle up to ~100 times [6].

The ubiquitously expressed TFR is a type II transmembrane homodimeric receptor which serves as the main route of entry for iron into most cells. Each of the ~90 kDa monomers of the TFR homodimer is comprised of a short cytoplasmic tail (residues 1–67) with an endosomal internalization motif, a single membrane-spanning portion (residues 68–88), a stalk region (residues 89–120) which contains two intermolecular disulfide bonds (Cys89 and Cys98) which covalently link the two monomers and a large extracellular ectodomain (residues 121–760) [7] [8]. The hTF binding TFR ectodomain also referred to as the soluble portion of the TFR or sTFR (residues 121–760) is further subdivided into three domains: the protease-like domain (residues 121–188 and 384–606), the apical domain (residues 189–383) and the helical domain (residues 607–760) [9]. A stoichiometric 2:2 complex is formed between hTF and the TFR such that each TFR monomer binds one molecule of hTF [10]. Importantly, even in the absence of the stalk region, the sTFR in solution appears to always exist as a dimer due to the strong interaction of the helical domains of each monomer with each other.

At any given time only 15–20% of the TFR molecules reside on the cell surface. An important and relevant question is whether the TFR is constitutively recycled or not, e.g., does the binding of hTF to the TFR promote internalization. The answer appears to be yes although this occurrence may be cell specific [11, 12]. Thus it appears that HeLa cells undergo constitutive recycling whereas internalization of TFR on HL60 cells and both human and rabbit reticulocytes is influenced by hTF binding.

It is well established that because of their characteristically high rates of proliferation, cancer cells have dramatically increased iron requirements and therefore express an increased number of hTF receptors. This may be related to the fact that the rate limiting enzyme in DNA synthesis is iron containing ribonucleotide reductase [13]. A recent extensive review provides a detailed description of the many strategies used to target the hTF/TFR system [14] and Figure 1. As with all chemotherapy, the aim is to target the malignant cells/tumor while minimizing any detrimental effects on the organism as a whole. As an obvious example of this conundrum, reticulocytes within bone marrow actively synthesize iron containing heme used to carry oxygen. The increased number of TF receptors on reticulocytes makes them particularly vulnerable to the hTF/TFR targeting strategies and is likely to result in anemia. In this short review we focus on the structural and functional requirements that should be considered when attempting to use the hTF/TFR system for targeted therapeutics.

2. hTF/TFR Specificity

Since the hTF/TFR interaction is critical to internalization and iron delivery within the cell, the molecular details of how the TFR interacts with its ligand, hTF, are clearly of great importance and must be carefully considered in developing hTF conjugates as targeted drug delivery vehicles. Until recently, a lack of precise structural details for the complex prevented an accurate consideration of the molecular interactions between hTF and the TFR. The first view of the TF/sTFR complex was a 7.5 Å resolution cryo-electron microscopy (EM) model published in 2004. Using the crystal structure of the unliganded sTFR and individually modeling in the available structures of the isolated human N-lobe and rabbit C-lobe (~85% similarity to the human C-lobe), this model suggested that the N-lobe is situated between the membrane and the TFR, and that the C-lobe makes significant contacts with the helical domain of the TFR (PDB ID: 1SUV) [15]. Due to the relatively low resolution and the ~9 Å shift with respect to the C-lobe required to fit the N-lobe into the existing density, this model provided a rather hazy picture of the interaction between hTF and the TFR. Availability of the structure of apohTF [16] and use of *in silico* modeling in conjunction with various mutagenesis studies of both hTF and the TFR resulted in a new model of hTF bound to the TFR [17]. When published, this computational model eliminated the physically impossible 9 Å gap between the N- and C-lobes of hTF, and was consistent also with the available mutagenesis data [2, 18].

2.1. Structure of the hTF/sTFR Complex

Publication of a relatively high resolution (3.22 Å) crystal structure of the Fe_NhTF/sTFR complex has now provided more precise molecular details of the interaction between the two binding partners [19]. Although this higher resolution structure is consistent with the relative orientation of the binding partners in the cryo-EM work, it provides considerably more detailed information about specific residues that are involved in the high affinity binding. The most important finding from this work is that the structure of the TFR undergoes significant changes as a result of the hTF binding. This was predicted by the Bjorkman group who had found that binding of the hemochromatosis protein, HFE to the TFR elicited structural changes in the TFR [20]. Because of the movements in the TFR when hTF binds, the cryo-EM model and the computational models based on the cryo-EM model have a number of inaccuracies with regard to the specific residues participating in the interaction. Nevertheless, similar to the cryo-EM model, three distinct binding motifs between hTF and the TFR are observed: the hTF N1-TFR motif, the hTF N2-TFR motif and the hTF C1-TFR motif. Obviously, covalent conjugation of a drug or toxin to residues involved in any of these three motifs could have detrimental effects on the hTF/TFR interaction and limit the actual amount of drug or toxin delivered to targeted cells. Furthermore, the interacting residues are surface exposed and therefore might be particularly susceptible to conjugation depending on the chemistry used.

2.1.1. hTF N1-TFR Motif—The hTF N1-TFR motif is predominately comprised of backbone-backbone interactions between residues in hTF and the TFR [19]. In a competitive immunoassay, the R50A Fe₂hTF mutant was unable to effectively compete with biotinylated Fe₂hTF for binding to immobilized sTFR; binding was reduced by 70% [21]. This significant decrease in the apparent binding affinity of the R50A hTF mutant for the sTFR demonstrated that the N1-TFR interaction is strengthened by the presence of the single salt bridge between Arg50 in hTF and Glu664 in the TFR [19] (Figure 1, panel B). Obviously, conjugation of a drug or toxin directly to or in the vicinity of Arg50 could affect the ability of an hTF/drug conjugate to effectively compete with native hTF for binding to the TFR on targeted cells.

2.1.2. hTF N2-TFR Motif—Two loops within the N2 subdomain make contacts with the TFR: the first loop, residues 139–145 (referred to as the PRKP loop) and the second loop, residues 154–167 [19]. Isothermal titration calorimetry (ITC) studies using alanine mutants of each of the individual residues in the PRKP loop (Pro142-Arg143-Lys144-Pro145) have clearly shown that three out of the four residues (Pro142, Lys144 and Pro145) participate in high affinity binding of hTF to the TFR [2]. In the competitive immunoassay format binding of the E141A and the K148A mutants was reduced by approximately half when competed against biotinylated Fe_2hTF for binding to immobilized sTFR [21]. It is difficult to clearly show these loops in the context of the TFR and crucial to understand that they move as a function TFR binding, lower pH and iron release. Again, covalent conjugation of a drug or toxin to residues in either loop could disrupt and significantly inhibit the ability of the hTF/drug conjugate to compete with native hTF for TFR binding.

2.1.3. hTF C1-TFR Motif—An extensive interface exists between the C1 subdomain of hTF and the TFR (Figure 1, panel C). While the cryo-EM model predicted a network of four salt bridges between the C1 subdomain and the TFR, only one between Asp356 in hTF and Arg651 in the TFR was identified in the $\text{Fe}_\text{N}\text{hTF/sTFR}$ crystal structure. This discrepancy is attributed to the fact that α -helix 1 in the C-lobe of hTF is shifted $\sim 5 \text{ \AA}$ (nearly one full helical turn) in the $\text{Fe}_\text{N}\text{hTF/sTFR}$ structure compared to the cryo-EM model [19]. Nonetheless, a number of charged hTF residues reside in the large C1 subdomain-TFR interface and contribute to the interaction. By mutating these residues in the C1 motif to alanine, we were able to evaluate the role of charged hTF residues in both binding to the sTFR and receptor-mediated iron release [21]. Through these studies, we identified Asp356 in the C-lobe of hTF as a residue that is absolutely essential to the formation of a stable hTF/sTFR complex. Disruption of the hTF Asp356-TFR Arg651 salt bridge, either by mutation or by conjugation of a drug/toxin, would significantly inhibit the ability of hTF to bind to the TFR. Moreover, although not all charged residue-to-alanine hTF mutants produced as dramatic an effect on binding as the D356A mutant, many of them reduced the binding of hTF to the sTFR. Specifically, the R352A, E357A, E357A/E625A, E367A, and K511A Fe_2hTF mutants were unable to effectively compete with biotinylated Fe_2hTF for binding to the sTFR immobilized on the assay plates with a significant reduction in binding (70% or more) [21]. Binding of one other alanine mutant, E385A Fe_2hTF was reduced by approximately half (Figure 1, Panel C). These results are indicative of some of the more subtle details of the hTF/TFR interaction. Additionally, since no iron is present in the C-lobe of the $\text{Fe}_\text{N}\text{hTF/sTFR}$ crystal structure, it is possible that the contacts between the C1 subdomain and the TFR are slightly altered/strengthened when iron is bound in that lobe. In support of this proposal, mutation of Lys511 in the C2 subdomain (which does not appear to make any contacts with the TFR) affected binding to the sTFR, further suggesting that this residue, possibly in conjunction with other residues within the C2 subdomain, stabilizes binding of the iron-bound C-lobe in the hTF/sTFR complex (Figure 1, Panel D).

2.1.4 Changes in the structure of the sTFR as a result of the binding of $\text{Fe}_\text{N}\text{hTF}$

hTF—To a large extent iron release from the hTF/TFR complex appears to be controlled by changes within the interface of the two TFR monomers and the C1 subdomain of hTF [22]. We have definitively established that this region is stabilized by a coordinated Ca^{2+} ion (held in place by two residues from the protease-like domain (Glu465 and Glu468) and three residues from the apical domain of the TFR (Asp307, Thr310 and Phe313)) and by the presence of carbohydrate attached to Asn317. Mutation of the two glutamine residues to alanine (E465A/E468A) to block Ca^{2+} binding or of Asn317 to Asp to prevent glycosylation both have a negative effect on protein production and behavior [22, 23]. As alluded to above, a key observation is that binding of hTF to the sTFR results in movement of a long loop (residues 275–338) in the apical domain within each TFR monomer interface. This

movement causes residue His318 to shift nearly ~ 18 Å in the Fe_NhTF/sTFR structure in comparison to the unliganded TFR structure [19] (Figure 1, Panel E). Additionally, at neutral pH His318 in one TFR monomer appears to interact with one or more of the four residues at the C-terminus of the other TFR monomer (residues 757–760). We suggest that exposure to the acidic environment within the endosome leads to protonation of His318, severing its interaction with the other TFR monomer C-terminus and allowing it to interact with other residues. Specifically, as indicated by a decrease in the fluorescent signal attributed to quenching of these residues by the protonated His 318 we propose that His318 interacts with Trp641 and/or Trp740 [22] (Figure 1, Panel E). A conformational change which promotes iron release from the C-lobe of hTF is triggered by the interaction between protonated hTF residue His349 and one or more of the four residues at the C-terminus of the TFR (residues 757–760). A potential salt bridge between Lys511 in the C2 subdomain and Glu372 in the C1 subdomain must be broken in the process (Figure 1, Panel C). These events in the C-lobe are communicated to the N-lobe of hTF, through helix α III-3 in the TFR to which both the N1 and C1 subdomains remain bound.

3. Targeting the hTF/TFR for chemotherapeutic treatments

A remarkable, and perhaps unprecedented, number of approaches have been used to target the TF/TFR pair separately or in concert. As mentioned, a recent review [14] thoroughly documents this enormous body of literature expanding on and updating earlier reviews [24–26]. Based on the well established fact that actively dividing malignant cells have higher numbers of the TFR, strategies include targeting TF to deliver a cytotoxic payload inside the cells or targeting the TFR with antibodies or peptides that interfere with the ability of TF to bind to the TFR and deliver the iron that is critical to continued proliferation. Payloads that have been conjugated to hTF using different chemistries include drugs (a large variety of alkylating agents), plant derived toxins, polyethylene glycol, RNases and nucleic acids. An alternate strategy is to create targeting carrier systems to encase the therapeutic agents listed above. These include micelles and liposomes, dendrimers, cyclodextrans, viruses, and single polymers or polymeric nanoparticles as detailed in the recent review by Daniels et al [14] and schematically illustrated in Figure 2.

3.1 Issues to consider in targeting hTF/TFR

In examining this enormous body of literature a number of issues emerge. Because normal serum naturally contains a high concentration of hTF (35 μ M), it is often difficult for hTF conjugates to out compete native hTF for binding to the TFR. A major concern is the dearth of quantitative measurements. Perhaps necessarily, most studies take an empirical approach, evaluating whether the effectiveness of the conjugate exceeds that of the drug alone and also whether the side effects are reduced or eliminated. Quite often it is unclear why promising approaches were not further pursued. To date, in this entire line of research targeting the TF/TFR system, only a single clinical trial has entered into Phase III; a mutant diphtheria toxin conjugated to hTF (designated TF-CRM107 or TransMIDTM) was used to treat patients with malignant brain tumors. However, as described in detail [27], the Phase III trials were cancelled when the results failed to significantly exceed the current “standard of care” for treatment of glioblastoma multiformes.

Since iron metabolism is intimately associated with both the immune system [28] and the nervous system [29, 30], the potential side effects on these systems must be considered with any therapeutic agent ultimately created for use in a clinical setting [31, 32]. Other issues include the presence of a second TFR designated TFR2 that is found largely in the liver. Although hTF binds to the TFR2 with somewhat lower affinity in comparison to TFR1 [33], any hTF conjugate could potentially increase damage to the liver [14]. For this reason it has been suggested that targeting the TFR1 directly using antibodies might prove to be a more

effective overall strategy since the two TFRs are only 45% identical and thus immunologically distinct.

3.2 Evaluation of TF conjugates

Within the broad outlines of the structural information described above a few key concepts should be considered when using this particular system to target and kill malignant cells. Some guidelines to establish a more uniform and practical approach to sample evaluation are suggested. The first step in determining the effect of conjugation on hTF should be to evaluate its properties including iron binding and/or release as well as its ability to compete with native hTF for binding to the TFR. It also seems critical to establish the homogeneity of a given conjugate since, as with any protein derivative, only a small fraction might be functional. Ideally, in terms of application to translational research the preparation of a given conjugate should be both predictable and reproducible. In this regard mass spectrometry is a powerful technique to evaluate the degree of homogeneity/heterogeneity. Recombinant hTF in which glycosylation has been eliminated by mutation immediately reduces the amount of heterogeneity and increases the accuracy of the analysis by mass spectrometry [5, 34].

Iron loaded hTF is distinguished by characteristic spectral properties which provide a unique signature. Thus binding of Fe^{+3} increases absorbance in both the UV and visible portions of the spectrum, attributed to energy transfer between Fe^{+3} and the two liganding tyrosine residues in each lobe which create a ligand to metal charge transfer band. The visible spectrum has a maximum centered near 466 nm resulting in the pink color of Fe_2hTF whereas apohTF is colorless [35]. Also the binding of iron to hTF quenches the intrinsic fluorescence attributed to excited state energy transfer from specific tryptophan residues in each lobe to the absorbance band [36, 37]. Thus, when iron is released the fluorescent signal increases and the absorbance decreases allowing determination of rate constants for iron release from each lobe [38]. The possible effect of conjugation on the visible spectrum of TF can be relatively easily ascertained by scanning the hTF conjugate in the visible part of the spectrum. At a basic level, if the conjugate interferes with the ability of hTF to retain iron it will obviously also affect the efficiency of delivery into the cell since TFR binding greatly favors iron loaded TF.

Alternatively, 6% urea gels provide a simple method to distinguish between the four possible forms of hTF with regard to iron content. Importantly, commercially available buffers and gels reagents have simplified the ease of use of this approach [39]. Because the migration of hTF through these gels is influenced by the iron content (which influences its shape and charge), as well as the disulfide bond content of the two lobes this technique could be used to indicate the heterogeneity of a conjugate. Predictably, as with mass spectrometry, recombinant hTF that is nonglycosylated increases the sharpness of the bands [39]. Furthermore, this approach requires only a small amount of sample (2–3 μg).

A more challenging but important parameter to measure is iron release. We recently provided a complete set of kinetic rate constants for release of iron from each lobe at the endosomal pH of 5.6 (both in the presence and absence of the TFR) [38]. These studies were made possible by the use of recombinant hTFs with predictable properties with respect to iron binding and/or release. Thus monoferric species, $\text{Fe}_\text{N}\text{hTF}$ and $\text{Fe}_\text{C}\text{hTF}$ (unable to bind iron in the other lobe) [40], as well as hTF molecules incapable of releasing iron from one or both lobes, provided accurate rate constants for each step in the alternate pathways leading from Fe_2hTF to apohTF [39]. However, this type of measurement is technically more challenging and requires a stopped-flow spectrofluorimeter which may preclude routine application to characterization of conjugates.

3.2.1 Binding studies to provide relative binding affinities of conjugates versus native TF

The ability of a given conjugate to bind to the TFR should be determined by quantitative binding studies. Techniques which have been used include: binding of radiolabeled hTF to cells either as a function of time or under equilibrium conditions, surface plasmon resonance (SPR), and isothermal titration calorimetry (ITC). Labeling of hTF with the radioisotope ^{125}I provides a sensitive and convenient way to measure binding to the TFR on cells as a function of time or of hTF concentration under equilibrium conditions in which iron uptake is inhibited (4°C or NH_4Cl). Addition of increasing amounts of $\text{Fe}_2^{125}\text{I}$ -hTF to a constant number of cells allows determination of both affinity constants and the number of TFR molecules per cell. Labeling of hTF with ^{59}Fe or ^{55}Fe allows iron uptake and in some cases incorporation into heme to be measured as a function of time. As recently discussed [6], such studies were routine in the early years of TF research and are difficult to match in terms of quantitative information provided. The simplest experimental approach involves competing increasing amounts of unlabeled hTF samples/mutants/conjugates against a constant amount $\text{Fe}_2^{125}\text{I}$ -hTF to derive a dissociation constant [34, 41]. Using this approach to measure binding of Fe_2hTF at neutral pH to TFR on Hela cells gave dissociation constants of 5.4, 10.4 and 6.6 nM on three different days with different batches of cells [41].

The only method available that is able, in theory, to provide all thermodynamic parameters in one experiment is ITC. A major advantage of ITC is that the “native” binding partners can be used, i.e., neither partner is altered by addition of labels or is tethered to a surface. A major disadvantage is that the technique requires considerably more material than radiolabeling or SPR (see below). The results of a recent ITC study in which the binding of Fe_2hTF at neutral pH to the soluble portion of the TFR was measured yielded a single apparent K_d of 4 ± 2 nM [2].

As recently reviewed in detail [42], the Bjorkman laboratory has carried out extensive SPR studies and analyzed the results in two different ways [33, 43]. In the first model, the data for Fe_2hTF binding to sTFR attached to a chip (through its His tag bound to an anti-His antibody) and tested at pH 7.5 was fit using a heterogeneous 2:2 model in which two classes of non-interacting binding sites on a TFR homodimer yielded two K_D s (1.9 nM and 29 nM). The second and favored method used the association and dissociation rate constants, k_a and k_d , CLAMP99 software and a 2:1 bivalent ligand reaction model, in which two hTF molecules bind sequentially to a single TFR homodimer. Using this approach K_{D1} and K_{D2} were reported to be 1.1 nM and 29 nM in one study [33] and 0.72 ± 0.6 and 4.1 ± 1.4 nM in a second study [18]. In contrast to the results from SPR, the other methods mentioned above yield a single dissociation constant. Of interest a single dissociation constant has been reported for the binding of Fe_chTF to the TFR [23].

Another particularly innovative strategy developed by the Kaltashov laboratory provides semi-quantitative data that may well be adequate to evaluate receptor affinity for hTF conjugates in comparison to the native hTF [5]. This approach uses mass spectrometry to determine the ability of a particular hTF species to displace another. Such competition experiments clearly confirmed that the iron status of hTF affects TFR binding affinity: $\text{Fe}_2\text{hTF} > \text{Fe}_N\text{hTF} > \text{Fe}_c\text{hTF} > \text{apo hTF}$. In combination with more conventional analysis by mass spectrometry this approach maximizes information content using modest amounts of sample.

More recently our laboratory designed a competitive immunoassay in a 96-well plate format in which wells were coated with rabbit anti-mouse IgG to capture an antibody that is specific to the sTFR [21]. A solution of sTFR is added to saturate all of the antibody binding sites; addition of a constant amount of biotinylated Fe_2hTF in the presence of or absence of

unlabeled Fe₂hTF standards allows generation of a standard curve. The ability of various mutant hTF samples to compete with the biotinylated Fe₂hTF allowed assessment of the effect of the mutation on binding to the sTFR [21]. Like the mass spectrometry approach, although this assay does not provide binding constants it does allow an evaluation of relative binding affinity which may be adequate for this particular purpose.

3.2.2 Experimental systems used in evaluation—Evaluation of the effectiveness of a given compound (the pharmacokinetics) necessarily involves a hierarchy of experimental systems usually starting with appropriate cell lines (both erythroid and non erythroid) derived from humans, mice or rats and then working up to whole animal models (usually rats or mice). A system in common use involves human tumors implanted into nude mice or rats [27, 44]. The challenges associated with translating the results of these kinds of studies across species are many. In general, the TF of a given species binds with highest affinity to the TFR of the same species, although some exceptions exist. As an extreme example bovine TF binds very poorly to the human TFR [45, 46]. Thus human cells cultured in fetal calf serum are starved for iron leading to the synthesis of more and more TF receptors; HeLa cells passaged in fetal calf serum typically have 1–2 million TFR/cell [45]. Therefore, species compatibility must be considered when attempting to utilize the hTF/TFR system as a drug carrier. It is important to evaluate and quantify the ability of a TF from one species to effectively compete with a TF of another species for binding to a given TFR in order to establish the validity of the results of such studies.

3.2.3 Recent innovations—A recent study beautifully illustrates many of the issues and challenges of attempting to target the TF/TFR and offers a clever solution [47, 48]. Access to the brain across the blood brain barrier (BBB) is extremely limited. Controversy abounds with regard to the selectivity of this barrier; some molecules are thought to enter the brain through simple diffusion, others through receptor-mediated endocytosis or by a process referred to as receptor-mediated transcytosis [49]. Whether TF itself or simply the iron from TF is transported into brain remains an area of some disagreement. What is clear is that the endothelial cells that comprise the BBB feature a large number of TF receptors. To capitalize on this fact, a bispecific antibody was created; one arm of this antibody has a variable region specifically designed to bind weakly to mouse TFR while the other arm was designed to bind with high affinity to and to inhibit the β -secretase enzyme which is responsible for plaque formation in Alzheimer's disease. The work clearly shows that the transport of the antibody into the mouse brain is significantly improved by targeting the mouse TFR. Specifically, uptake of non-targeted antibodies is dose dependent with an upper range of only 0.2% [49]. Likewise the low affinity binding to the mouse TFR resulted in the release and wide distribution of the chimeric antibody once in the brain.

Another innovative approach involves combining mathematical modeling with recombinant technology to design and utilize hTFs with desirable properties [27, 42, 50]. Specifically mutants which lock the iron in each site could potentially increase the dwell time within the cell and/or recycle more efficiently since it remains iron loaded.

It is of interest and relevance to point out that the TFR has been harnessed during evolution to efficiently transport some New World viruses inside cells [51]. Thus certain New World arenaviruses (Machupo, Junin, Guanarito and Sabia) that cause hemorrhagic fevers and often result in death specifically target the exposed tip of the TFR apical domain. The particular binding site is distant from the hTF binding sites described above. In this manner the viruses hitch a ride into cells where the lower pH of the endosome releases them to replicate. The authors suggest that these arenaviruses “fortuitously” developed affinity for the human TFR to become pathogenic and that antibodies that target the same portion of the apical domain of the TFR might be useful in treatment.

4. Conclusion

In summary, since its discovery in the 1980s, the hTF/TFR pair has served as *the* classic example of receptor mediated endocytosis assuring the safe delivery of redox active Fe³⁺ into actively dividing cells in a pH specific manner. The specificity and high affinity of the interaction between hTF and the TFR have attracted unprecedented attention as an obvious target to transport and deliver all manner of therapeutic agents to the inside of cells [14]. Although it is clear that this particular Trojan horse approach has much to offer, the full potential remains largely unrealized. To date, the perfect combination of specificity and lethality has not been found. We suggest that a more careful examination of the structural aspects of the interaction might provide a more focused less shotgun approach to rational drug design and utilization of this intriguing system.

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Abbreviations

apoTF	serum transferrin that is iron free
BBB	blood brain barrier
EM	electron microscopy
Fe_ChTF	recombinant N-terminal hexa-His tagged non-glycosylated monoferric hTF that binds iron only in the C-lobe
Fe_NhTF	recombinant N-terminal hexa-His tagged non-glycosylated monoferric hTF that binds iron only in the N-lobe
Fe₂hTF	recombinant N-terminal hexa-His tagged non-glycosylated diferric hTF
HFE	the hemochromatosis protein
hTF	human serum transferrin
scFv	single chain antibody variable region
SPR	surface plasmon resonance
sTFR	glycosylated N-terminal hexa-His tagged soluble recombinant transferrin receptor (residues 121–760)
ITC	isothermal titration calorimetry
TFR	transferrin receptor

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Figure 1.

A. The structure of diferric human diferric serum TF (PDBID 3V83) was least square fitted (left RMS = 1.183Å & right RMS = 1.007Å) on the Fe_NhTF/TFR structure (PDBID 3S9N) to generate a model of the diferric TF/TFR complex. The different shades of blue indicate the two TFR monomers which comprise the TFR dimer; the different shades of yellow indicate the two TF molecules (one each binding to each TFR monomer). Colored boxes indicate where panels B–E are located in the model. Green spheres in the TFR indicate the positions of calcium ions and red spheres show the position of the ferric ions in lobe of TF. Panel B: Closeup of the N1 TFR motif (PDBID 3S9N). Blue = TFR and yellow = TF. Panel C: Closeup of the C1 TFR motif (PDBID 3S9N). Blue = TFR and yellow = TF. Panel D: Closeup of the C2 TFR motif using the diferric transferrin/receptor model (Panel A). All figures were produced using PyMOL. Blue = TFR and yellow = TF. Again, the red sphere shows the position of ferric ion. Panel E: Least squares fit of the TFR alone (bright pink, PDBID 1CX8) and the Fe_NTF/TFR/structure (steel blue, PDBID 3S9N) (RMS = 1.000Å). This figure illustrates the large movement (~18Å) of the loop containing His318 which flips down as a result of the binding of Fe_NTF to the TFR. Additionally the positions of TFR residues Trp641 and Trp740 are shown.

Figure 2.

Cartoon of some strategies used to target therapeutic agents to the TFR on cancer cells. This figure has been adapted with permission from and thanks to Daniels et al. [14] and provides an overview of some of the approaches that have been taken. As shown, strategies include attachment of cargos directly to TF, or to peptides that bind to the TFR, in addition to monoclonal antibodies or single chain antibody fragments (designated scFv) specific for the extracellular domain of the TFR. The therapeutic agents are delivered either as direct conjugates or enclosed in carriers. Therapeutic payloads include chemotherapeutic peptides, small drugs, proteins, radionuclides, genes in vectors, and oligonucleotides. The TFR is shown as a homodimer; importantly each monomer of the TFR is capable of binding a molecule of TF (although for clarity only the binding of a single TF is shown). Likewise for simplicity in the antibody and related scFv targeting sections a single TFR homodimer is shown in the cartoon.