TITLE

Transferrin and the transferrin receptor for the targeted delivery of therapeutic agents to the brain and cancer cells

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SUMMARY

The potential use of many promising novel drugs is limited by their inability to specifically reach their site of action after intravenous administration, without secondary effects to healthy tissues. In order to remediate to this problem, the protein transferrin has been extensively studied as a targeting molecule for the transport of drug and gene delivery systems to the brain and cancer cells. A wide range of delivery approaches have been developed to target the transferrin receptor and have already improved the specific delivery of transferrin-bearing therapeutic agents to their site of action.

This review provides a summary of the numerous delivery strategies used to target the transferrin receptor and focuses on the recent therapeutic advances being made so far.

KEY TERMS

Transferrin; delivery system; tumor targeting; brain delivery; blood-brain barrier

INTRODUCTION

Recent advances in the fields of pharmacology and chemistry have led to the discovery of numerous potential drugs with promising therapeutic effects on various pathologies. However, the clinical use of these drugs following intravenous administration is currently limited by the lack of specific delivery to the pathological site, resulting in toxicity and secondary effects to healthy tissues. In order to remediate to this problem and enhance the therapeutic index of the drugs, it is crucial to target these drug candidates to their site of action.

For more than twenty years, the protein transferrin (Tf) has been extensively studied as a targeting moiety for drug and gene delivery systems, since it is non-toxic, non-immunogenic and biodegradable [1]. Transferrin receptors (TfR) are highly expressed on the brain capillary endothelium which forms the blood-brain barrier (BBB) in conjugation with other cellular components such as pericytes and astrocytes, *in vivo* [2]. As intravenously administered Tf was shown to reach the brain by receptor-mediated transcytosis across the BBB [3,4], TfR-targeting strategy quickly became the object of intense investigation for the brain delivery of central nervous system drug candidates: 100% of large-molecule drugs and more than 98% of small molecules drugs are otherwise prevented from penetrating the brain by the BBB [5]. In addition, TfR are present in abundance on the surface of cancer cells [6-9], thus allowing a specific uptake of TfR-targeted therapeutic drugs and genes by cancer cells overexpressing transferrin receptors.

This review has for objective to present in a succinct form the numerous TfR-targeting strategies developed so far in laboratory settings, while focusing on recent promising breakthroughs in this research area.

1. Transferrin and transferrin receptors: generalities

1.1. Transferrin: structure and function

Transferrin is part of a family of ubiquitous iron-binding glycoproteins including ovotransferrin, lactoferrin and melanotransferrin, whose primary function is the binding and transport of iron [10,11]. It is a monomeric glycoprotein containing about 700 amino acids, with a large molecular mass (approximately 80 kDa). The polypeptide chain is folded into 2 structurally similar globular domains, known as the N- and C- lobes, and connected by a short linear peptide linker [11]. Each lobe contains one binding site for Fe^{3+} with a very high affinity (10^{22} M^{-1} at pH 7.4) [12]. The iron-free transferrin (apotransferrin) can therefore bind up to 2 Fe^{3+} ions to form ferro-transferrin (also called holotransferrin). The conformational changes of the protein occurring during iron binding have been shown to play a key role in the selective recognition by TfR. Consequently, diferric Tf has a 10 to 100-times higher affinity for the TfR than that of apoTf at physiological pH [13] and is therefore preferred as a TfR ligand in targeting studies.

The principal biological function of Tf is to bind and distribute iron in the body. Tf is the main iron-transporting protein in the body. It binds circulating iron taken from the diet and transports it into the systemic circulation to various target tissues. This transport is extremely important, as iron is required for various biological processes such as DNA synthesis, oxygen transport and electron transfer [11].

Due to its iron binding mechanism, Tf also protects the cells against the toxic effects that could be generated by the presence of free iron in the circulation. It is only up to 30-40% iron-saturated under physiological conditions [14].

In addition to its key role of iron transporter, Tf has been shown to bind other cations such as copper, gallium, zinc, manganese and aluminium ions [10].

1.2. Transferrin receptor

Cellular uptake of iron-loaded transferrin occurs through receptor-mediated endocytosis. The Tf receptor, referred to as TfR1 or CD71 in literature, is a type II transmembrane glycoprotein involved in iron uptake and cell growth regulation [15]. It consists of 2 identical monomers with an approximate molecular mass of 90 kDa each, linked by 2 disulfide bonds [1]. Each monomer is made of 3 distinct regions: a short N-terminal cytoplasmic domain, a hydrophobic transmembrane domain and a large, globular, extracellular C-terminal domain which contains the binding site for Tf [1,16]. As each monomer may bind a molecule of Tf, up to 2 molecules of Tf may bind one TfR and up to 4 Fe^{3+} ions within one Tf-TfR complex can be taken up by the cell. The recycling rate of TfR1 is very short (about 10-20 s) and can occur hundred times for each single receptor [53], making this receptor particularly efficient for the delivery of iron and therapeutics to cells.

Another member of the Tf receptor family, referred to as TfR2, has been discovered. Although its extracellular domain shows 66% similarity with TfR1, TfR2 has a much lower affinity for Tf than TfR1 (25-fold lower) [17]. In addition, its expression is not correlated to iron levels in the cells. Its α-transcript product is mostly expressed on hepatocytes, while its β-transcript is present on a wide range of tissues but at very low levels [17]. TfR2 would therefore be a less efficient target for transferrin-mediated drug and gene delivery to the brain or cancer cells.

TfR1 is expressed at low levels in most human tissues, but is highly expressed on the vascular endothelium of brain capillaries [2]. It is also expressed at levels up to 100-fold higher than those on normal cells on highly proliferative cells such as cancer cells [6-9]. Its increased expression on tumors is generally correlated with cancer progression and tumor stage [6-9].

This high expression of TfR on brain capillaries and cancer cells, together with the ability of the receptor to internalize with its ligand, makes this receptor a highly promising target for the delivery of therapeutics to the brain and cancer cells.

2. Transferrin receptor-mediated delivery of therapeutic agents to the brain

Most therapeutic molecules, including small molecule drugs, proteins, peptides and genes, cannot reach the brain after intravenous administration due to their inability to cross the BBB [4,5]. As a result, various drugs with promising therapeutic effects *in vitro* are found to be ineffective for the treatment of neurological diseases such as Parkinson's and Alzheimer's diseases as well as brain neoplasms.

Widely expressed on the BBB, TfR has been widely investigated for the delivery of drugs and genes to the brain. The strategy employed here would be to use Tf or antibodies directed against TfR as TfR-targeting moieties. Therapeutic agents entrapped in TfR-targeted delivery systems would then be able to cross the BBB and to be delivered into the brain.

2.2. Targeted drug delivery

Many drugs are unable to cross the blood-brain barrier. In order to overcome this problem, therapeutic molecules have been encapsulated in delivery systems able to recognize TfR and to deliver their cargo to the brain. A wide range of delivery strategies, including the linkage of drugs to Tf/ anti-TfR antibody, complexation with TfR-targeted quantum rods, entrapment of the drugs in TfR-targeted liposomes, lipid nanocapsules, nanoparticles and micelles, have been investigated for TfR-mediated delivery of drugs to the brain **(Figure 1).**

2.2.1. Linkage of drugs to Tf/ anti-TfR antibody

Therapeutic agents can be delivered across the BBB following their linkage to either the Tf or a molecule able to specifically recognize the TfR.

OX26, a mouse monoclonal antibody against the rat TfR1, has been explored as a drug delivery agent to the brain. It was used as an alternative to Tf itself in order to

prevent any eventual vector failure linked to a saturation of Tf receptors [18]. This antibody presents the advantage of binding to an extracellular domain for the TfR different from the Tf binding site and is therefore independent from Tf binding. It has been thoroughly studied for the brain delivery of numerous drugs following intravenous administration [1,4-5]. For example, it was conjugated to the hydrophilic anti-cancer drug methotrexate by a hydrazone bond. Approximately six molecules of methotrexate were attached per antibody. After intravenous injection to rats, the conjugate was shown to be able to cross the blood-brain barrier via TfR-mediated transcytosis and to deliver the drug to the brain, as confirmed by immunohistochemical detection of the conjugate in the brain parenchyma [19].

In another example, the brain-derived neurotrophic factor (BDNF), a neuroprotective agent, cannot cross the BBB after intravenous administration and is unable to exert its neuroprotective effect in the first hours after regional brain ischemia, because the BBB is still intact at this stage. However, the intravenous administration of OX26 conjugated to BDNF resulted in a 243% increase in motor performance of rats presenting stroke symptoms in comparison to the drug alone [20].

OX26 has also been used to deliver many other drugs, such as neuropeptides (vasoactive intestinal peptide) [21], proteins (nerve growth factor) [22], to the rat brain after intravenous administration. As OX26 is specific to the rat TfR, another monoclonal antibody anti-mouse TfR, named 8D3, has been used as a TfR1-targeting vector in the mouse [18]. This antibody, conjugated to radiolabeled $A\beta^{1-40}$, has been used to visualize and quantify β-amyloid plaques in a mouse model of Alzheimer‘s disease [23]. A chimeric form of the 8D3 antibody was also conjugated to the glial-derived neurotrophic factor (GDNF), a neurotrophin with therapeutic potential on Parkinson’s disease. The GDNF-antibody fusion protein was able to

cross the BBB after intravenous injection and was found to be therapeutically active in three neurobehavior models of experimental Parkinson's disease in mice [24].

Various approaches have been developed for the linkage of the drugs to Tf or anti-TfR antibodies. Among them, the avidin/biotin technology appears to be particularly promising for the delivery of drugs to the brain. The non-covalent binding between avidin and biotin is extremely strong (Kd~ 10^{-15} M) and stable in the circulation, but is labile in the tissues [25], thus facilitating the release of the drug once it reaches the brain. The avidin/biotin strategy was for example used to link OX26 and the vasoactive intestinal peptide (VIP), and resulted in an increase of cerebral blood flow following intravenous administration of this complex to rats [21].

This approach has also been successful in delivering high molecular weight-enzymes to the brain. For example, the intravenous administration of 116 kDa-β-galactosidase linked to the 8D3 monoclonal antibody via streptavidin-biotin binding resulted in a 10-fold increase in brain uptake of the enzyme, without loss of activity, in a mouse model [26]. In this study, brain delivery has been confirmed by brain capillary depletion method and brain histochemistry.

2.2.2. TfR-targeted quantum rods

Quantum rods are semiconductor nanoparticles widely used as luminescent probes for various biological applications. In addition to their well-known optical properties, they can be functionalized and carry therapeutic molecules. For example, Tf-conjugated quantum rods complexed to the anti-retroviral drug saquinavir were successfully transported across an *in vitro* BBB model through transferrin receptor-mediated transcytosis [27,28].

2.2.3. TfR-targeted liposomes and lipid nanocapsules

TfR-targeted liposomes are self-assembling vesicular structures based on one or more lipid bilayers encapsulating an aqueous core and able to recognize the Tf receptor. They can encapsulate hydrophilic drugs in their aqueous core or entrap lipophilic drugs in the lipid bilayer membrane. The use of conventional liposomes for brain drug delivery was initially limited by their size (which has to be smaller than 200 nm in diameter to allow drug delivery across the BBB [29] and by their rapid clearance by the reticuloendothelial system [30]. However, the latter problem was overcome by incorporating polyethylene glycol (PEG)- derivatized lipids in the lipid bilayer of the liposomes or by coating them with PEG, resulting in the formation of sterically stabilized (also called stealth) liposomes.

Tf-bearing liposomes have been used for the cerebral delivery of the anti-cancer drug 5-fluorouracil. The intravenous administration of this therapeutic system resulted in a 17-fold increase in the brain uptake of the drug compared to that was observed with the free drug, as demonstrated by biodistribution studies with the radiolabelled drug [31].

Antibody-directed liposomes, or immunoliposomes, have also been shown to be promising vectors for the targeted delivery of drugs to the brain. OX26 antibody was conjugated to PEG-coated liposomes encapsulating the anti-cancer agent daunomycin, which has a very low permeability through blood brain barrier. Brain delivery of OX26-bearing liposomes was higher than that of the non-targeted stealth liposomes, conventional liposomes or free daunomycin, following intravenous administration of the formulations to rats, as demonstrated by pharmacokinetic analysis [32].

Another lipid-based vector for brain targeting, called lipid nanocapsule, has recently been developed as a strategy to protect the entrapped drug from chemical and

enzymatic degradation. This nanovector is made of a hydrophilic surfactant, Solutol® HS15, surrounding an oily core [33]. The conjugation of OX26 antibody to the surface of the lipid nanocapsules doubled their concentration in the brain compared to non-targeted formulations, 24h after intravenous administration to rats [34].

2.2.4. TfR-targeted nanoparticles

Nanoparticles are polymeric colloidal spheres made of natural or artificial polymers, and ranging in size from 10 to 1000 nm. They can entrap drugs in their matrix, encapsulate them in their core or carry them on their surface. Like liposomes, they can be coated with PEG to prevent their clearance by the reticuloendothelial system.

Nanoparticles made of poly (butyl cyanoacrylate) (PBCA) or poly (d,l-lactide-co-glycolide acid) (PLGA) are among the most frequently used nanoparticle formulations for drug delivery to the brain [35-38]. For example, Tf-bearing PLGA nanoparticles entrapping the analgesic tramadol exhibited anti-nociceptive effects in rats for 24 h following their intravenous administration [38]. Similar results were obtained in rats following intravenous treatment with OX26-bearing PLGA nanoparticles entrapping endomorphins [37].

In another study, gold nanoparticles linked to the peptide CLPFFD had the capacity to destroy the aggregates of β-amyloid, similar to the ones found in the brains of patients suffering from Alzheimer's disease, but were unable to cross the BBB. Their conjugation to a peptide sequence recognizing the TfR allowed them to cross the BBB and to reach the brain of rats following intravenous administration, as demonstrated by quantification of gold in brain tissue [39].

2.2.5. TfR-targeted hybrid micelles

Micelles are small spherical structures with a hydrophilic surface and a hydrophobic core region. They recently attracted a lot of interest for the delivery of drugs to the brain due to their small size and ease of preparation [40]. A polyphosphoester hybrid micelle, consisting of Tf-conjugated PEG-poly(ε-caprolactone) and the amphiphilic block copolymer poly (ε-caprolactone)-block-poly(ethyl ethylene phosphate), was used to encapsulate the BBB-impermeable anti-cancer drug paclitaxel [41]. This Tf-bearing formulation enhanced intravenous delivery of the drug to the brain by 1.8-fold when compared to the non-targeted micelles and resulted in an improved anti-glioma activity in mice bearing intracranial glioma [41,42]. In these studies, only Tf-bearing formulations entrapping paclitaxel led to significant therapeutic effects on orthotopic glioma, thus rejecting the hypothesis that the delivery of the anti-cancer drug to the tumors might have been facilitated by the leakiness of tumor vessels.

It is also important to note that the therapeutic effect of a hydrophobic drug, which is often used as a measure for drug delivery efficiency, may be explained by binding of the drug carrier system to the brain endothelium or its uptake by endothelial cells, followed by diffusion of the drug further into the brain parenchyma. It does not necessarily mean that the drug carrier system crossed the BBB. The use of non-targeted delivery systems as controls is therefore highly important to confirm the TfR-mediated BBB crossing.

2.3. Targeted gene delivery

Numerous brain-related diseases could be candidates for treatment by gene therapy. Unfortunately, the inability of the nucleic acids to cross the BBB limits their therapeutic applications. In addition, a widespread expression of the exogenous gene

throughout the entire brain would be needed for the treatment of most cerebral disorders.

Viral delivery systems have been widely used for gene delivery, as they facilitate highly efficient transfer and expression of exogenous genes, but they can be immunogenic, cytopathic or recombinogenic, thus limiting their repeated administrations [43]. On the other hand, non-viral delivery systems have been explored as promising alternatives for gene delivery. Various approaches have been used to formulate TfR-targeted non-viral gene medicines able to transport nucleic acids to the brain following intravenous administration **(Figure 1).**

2.3.1. TfR-targeted liposomes

Various formulations of TfR-targeted liposomes have been investigated for the delivery of nucleic acids across the BBB.

Plasmid DNA encoding β-galactosidase has been delivered to the rat brain following its encapsulation in OX26-bearing stealth liposomes [44]. This delivery system crossed the BBB via transferrin receptor-mediated transcytosis and led to gene expression deep within brain parenchyma. Some levels of gene expression were also observed in the liver and the spleen [44].

This targeting strategy has also demonstrated efficacy in an experimental model of Parkinson's disease (PD). In order to remediate to the deficiency in striatal tyrosine hydroxylase (TH) characteristic of the disease, PD rat models were intravenously injected with TfR-targeted liposomes carrying a TH expression plasmid. This administration led to a normalization of striatal TH immunoreactivity. By contrast, rats treated with non-targeted liposomes did not show any improvement of their pathology, thus highlighting the importance of TfR for brain delivery [45].

TfR-targeting can also be used for the brain delivery of RNA interference (RNAi) in brain cancer therapy. One of the therapeutic strategies used in this instance was to knock-down the human epidermal growth factor (EGFR) which has a pro-oncogenic function in tumor cell growth and is overexpressed on tumors. The plasmid used encoded a RNA antisense to a specific region of the human EGFR mRNA. The liposomes encapsulating the nucleic acid were conjugated to an antibody anti-TfR to cross the BBB and to an antibody anti-human insulin receptor to reach the tumor once inside the brain. Intravenous administration of the formulation every week in mice with U87 intra-cranial brain tumors resulted in a 100% increase in survival time of the animals [46].

Similar experiments were also done in mice, using the rat 8D3 monoclonal antibody against mouse TfR instead than OX26 that specifically recognizes rat TfR. 8D3-conjugated PEGylated liposomes encapsulating plasmid DNA encoding β-galactosidase were able to deliver their cargo to the brain of mice following intravenous administration. When the enzyme expression was driven by a brain specific promoter, such as the glial fibrillary acidic protein promoter, gene expression was only observed in the brain, especially in the astrocytes, but not in any peripheral organs [47].

2.3.2. TfR-targeted conjugates/complexes

Another strategy to deliver nucleic acid therapeutics to the brain is to attach them to TfR-targeting ligands with the use of the avidin-biotin technology. This approach allowed Suzuki and colleagues [48] to image endogenous gene expression in the brain with sequence-specific antisense radiopharmaceutics. In this study, the biotinylated peptide nucleic acids were antisense agents to the rat glial fibrillary acidic protein (GFAP) mRNA or the rat caveolin-1α (CAV) mRNA. They were chelated to a

radionuclide and bound to a conjugate of streptavidin and OX26. The intravenous administration of TfR-targeted GFAP antisense to rats bearing brain glial tumors did not image brain cancer, due to the down-regulation of GFAP mRNA in cancer. However, TfR-targeted CAV antisense did lead to a selective imaging of brain cancer as a result of the administration of the delivery system, owing to the up-regulation of CAV mRNA in brain glial tumors [48].

Using the same avidin-biotin approach, the intravenous administration of a TfR-targeted siRNA led to 69-81% decrease in luciferase gene expression in brain cancer [49].

Dendrimers have recently been shown to be promising candidates for brain delivery, owing to their unique polymer architecture and easily modified surface groups. They are synthetic polymeric macromolecules of nanometer size, composed of multiple branched monomers that emerge radially from the central core [50]. Due to their unusual structure, dendrimers are characterized by the following properties that differentiate them from other polymers and make them attractive for gene delivery: monodisperse size, modifiable surface functionality, multivalency, water solubility and available internal cavity eventually suitable for drug delivery [50].

So far, only one dendrimer, polyamidoamine (PAMAM), has been studied as a vehicle to cross the BBB via TfR targeting [51]. In this study, transferrin was conjugated to PAMAM through bifunctional PEG and complexed to a plasmid DNA encoding green fluorescent protein (GFP). After intravenous administration to mice, the Tf-bearing PAMAM-DNA complex was able to cross the BBB and led to the expression of GFP in several brain regions such as the hippocampus, *substantia nigra*, the 4th ventricle, the cortical layer and *caudate putamen*. This gene expression

was about 2-fold higher than that observed following the administration of PAMAM and PAMAM-PEG complexes [51].

In other studies, a dendrigraft, poly-L-lysine, was developed as a delivery system for the co-administration of the anti-cancer drug doxorubicin and a therapeutic plasmid DNA encoding TNF-related apoptosis-inducing ligand (TRAIL) for brain tumor therapy. The combination of gene therapy and chemotherapy within one TfR-targeted delivery system was hypothesized to provide a synergistic therapeutic effect for the treatment of glioma. The peptide HAIYPRH (T7) was used as a TfR-targeting ligand. Doxorubicin was covalently conjugated to the dendrigraft via a pH-triggered hydrazine bond, while the DNA was complexed to the carrier. Following intravenous administration to mice bearing orthotopic glioma tumors, the TfR- targeted system was shortly detected in the brain and in the glioma (within 1h post-administration). The combination treatment then resulted in a synergistic growth inhibition of the tumors, with a survival time increased by about 20 days compared to controls [29].

Most studies described above demonstrated that their TfR-targeted delivery systems were able to cross the BBB by TfR-mediated transcytosis by showing that non-targeted delivery systems were not able to do so. The proof of such transport mechanism could also be obtained either by capillary depletion method or morphological investigation of brain tissue.

3. Transferrin receptor-mediated delivery of therapeutic agents to cancer cells

Traditional chemotherapeutic drugs can successfully eradicate tumors, but often have their use limited due to their non-specific toxicity to normal cells. To overcome this major problem, many tumor-targeting strategies are currently under development [52]. Among them, TfR targeting has been the object of intense research in the field of cancer therapy, owing to the overexpression of this receptor on malignant cells that can be up to 100-fold higher than that observed in normal cells [6-9]. This overexpression of TfR on cancer cells is much higher than that observed on the BBB and therefore limits any delivery of chemotherapeutics to the brain instead to tumors. Furthermore, TfR-targeted delivery systems were shown to be highly useful for the systemic treatment of brain tumors, as they were able to transport therapeutic agents across the BBB and to increase their delivery to brain tumors following intravenous administration, as further described below [29,49].

3.1. Targeted drug delivery

A wide range of delivery strategies, including the linkage of drugs to Tf, entrapment of the drugs in TfR-targeted liposomes, vesicles and nanoparticles, have been investigated for TfR-mediated delivery of drugs to tumors **(Figure 1).**

3.1.1. Linkage of drugs to Tf

Many anti-cancer drugs have been conjugated to either Tf or an antibody against TfR. One of them, doxorubicin, is an effective and widely used anti-cancer anthracyclin drug which exhibits severe adverse effects, such as cardiotoxicity, nephrotoxicity, myelosuppression due to non-specific drug distribution, and emergence of drug-resistant cancer cells. To overcome these limitations, doxorubicin was conjugated with Tf by glutaraldehyde cross-linking. This resulted in an increase

of the *in vitro* cytotoxicity of the conjugate, with an IC_{50} decrease of up to 57-fold compared to the free drug in L929 murine fibrosarcoma cells [53]. *In vivo* studies on nude mice bearing H-MESO-1 tumors demonstrated that the intravenously-administered Tf-Doxorubicin conjugate significantly decrease the size of the tumors and consequently increased the life span of the animals by 69%, compared to 30 % following administration of doxorubicin solution [54].

Conjugation of doxorubicin to Tf could also overcome the resistance of some cancer cells to doxorubicin solution, for example in KB human oral carcinoma cells and MCF-7 human breast cancer cells [55]. The resistance to free doxorubicin could be further overcome by conjugating Tf-doxorubicin conjugate with the antineoplastic drug gallium nitrate, which is a competitive inhibitor of the circulating holoTf. The Tf-doxorubicin-gallium nitrate conjugate was able to decrease the IC_{50} by 100-fold in doxorubicin-resistant MCF-7 human breast cancer cells [56].

Tf has also been conjugated to cisplatin, an alkylating agent used for treating testicular, ovarian and bladder cancers. The resulting conjugate was able to avoid metastatic growth of breast carcinoma cells in the lungs during *in vivo* experiments on rats. In addition, a clinical trial for advanced breast cancer has shown a high response rate to the Tf-cisplatin conjugate in 4 out of 11 patients, including one complete response, with only minor side effects [57].

3.1.2. TfR-targeted liposomes and vesicles

Encapsulating an anti-cancer drug in Tf-bearing liposomes or other lipid-based vesicles can lead to an increase in anti-proliferative efficacy and reduction of non-specific adverse effects. The intravenous administration of Tf-bearing liposomes encapsulating doxorubicin in nude mice bearing HepG2 human hepatoma

subcutaneous tumors, resulted in an increase of drug concentration in tumors and a subsequent decrease of tumor growth compared to doxorubicin solution [58].

Liposomes can also be conjugated to anti-TfR monoclonal antibodies instead of Tf. Such a strategy was used by Suzuki and colleagues [59], who reported the efficacy of doxorubicin-encapsulating liposomes conjugated to the anti-human TfR monoclonal antibody OKT9, on K562/ADM human leukemia cells resistant to free doxorubicin. In this *in vitro* study, TfR-targeted doxorubicin was taken up by the cells, thus resulting in an increased cytotoxic activity compared to the drug solution.

Tf-bearing palmitoyl glycol chitosan-based vesicles were also tested for the delivery of doxorubicin to A431 human epidermoid carcinoma and the drug-resistant A2780AD human ovarian carcinoma cells [60]. Increased cellular uptake and cytotoxicity were observed for Tf-bearing formulations as compared to doxorubicin solution. *In vivo*, however, none of the vesicular formulations could significantly delay the growth of A431 tumors after a single administration and were less active than the drug solution.

Tf-bearing vesicles were also found to be extremely useful to evaluate the therapeutic efficacy of promising anti-cancer drugs unable to reach the tumors at therapeutic concentrations. This was the case for example for tocotrienol, a member of the vitamin E family of compounds. We have demonstrated that the entrapment of tocotrienol within Tf-bearing vesicles led to tumor regression and increase of the animal survival following intravenous administration to mice bearing A431 tumors. [61]. In a follow-up study, we showed that intravenously administered Tf-bearing multilamellar vesicles entrapping tocotrienol led to complete tumor eradication for 40% of B16-F10 murine melanoma and 20% of A431 tumors, as well as improvement of animal survival [62,63]. A similar strategy allowed us to demonstrate that treatment with the green tea extract epigallocatechin gallate encapsulated in Tf-

bearing vesicles resulted in tumor suppression of 40% of A431 and B16-F10 tumors following intravenous administration of the targeted formulation [64].

3.1.3. TfR-targeted polymers and nanoparticles

Polymers and nanoparticles have become promising drug delivery systems for cancer therapy, due to their multifunctional structure that allows the simultaneous transport of various drugs to cancer cells, leading to synergistic therapeutic effects *in vivo*. Polymers used as single polymeric systems or as part of nanoparticles are also biodegradable and non-toxic. They can be of natural origin, such as the polysaccharide chitosan, or synthetic, such as poly (lactic acid) and poly (lactic-co-glycolic acid) (PLGA). TfR-targeting nanoparticles could be formulated to allow various kinetics of drug release depending of the pathology to be treated. For example, a slowly releasing polymer could be more advantageous for cancer treatment whereas a faster release may be required for acute brain disorders [65].Tf-bearing PLGA-based nanoparticles loading the mitotic inhibitor paclitaxel increased the delivery of their cargo to C6 rat glioma subcutaneous tumors compared to the non-targeted vehicles, after 24h of intravenous administration to rats [66]. In another study, 50% of treated S-180 tumor-bearing mice showed a complete tumor regression following the intravenous administration of Tf-bearing PEGylated poly(cyanoacrylate) nanoparticles entrapping paclitaxel. The life span of the animals was significantly increased, with 3 mice surviving over 60 days post-treatment with the TfR-targeting nanoparticles [67].

3.2. Targeted gene delivery

The possibility of using genes as medicines to treat patients suffering from cancer is still hampered by the lack of safe carrier systems able to specifically deliver the

therapeutic nucleic acids to the tumors after intravenous administration. There is therefore an urgent need to develop efficacious gene delivery systems that should be target-specific, non-toxic, non-inflammatory, biodegradable and non- immunogenic.

Among all the delivery systems currently being developed, non-viral delivery systems appear most suited to fulfill these requirements. Moreover, they present the advantages of being easy to prepare, flexible regarding the size of nucleic acid to be carried and suitable for repeated administrations as they are much safer than viral vectors [68]. TfR-mediated gene delivery using these vectors is a promising strategy since it provides an opportunity to achieve specific gene delivery to tumors while increasing the transfection efficiency in cancer cells **(Figure 1).**

3.2.1. TfR-targeted liposomes

Cationic liposomes complexed with DNA have been used extensively for the delivery of nucleic acids to cancer cells *in vitro* and *in vivo*. The negatively charged DNA can be complexed to the positively charged liposomes by electrostatic interactions to form lipoplexes. The intravenous injection of a Tf-bearing lipoplex encoding for the tumor suppressor protein p53 resulted in high level of p53 gene expression in tumors, contrarily to what observed with non-targeted lipoplexes, in mice bearing subcutaneous DU145 human prostate tumors [69]. In combination with radiotherapy, the TfR-targeting lipoplex encoding p53 exhibited complete tumor regression without recurrence 6 months after treatment [69].

3.2.2.TfR-targeted cyclodextrins

Cyclodextrins are a family of cyclic macromolecules consisting of 6-8 glucopyranoside units linked together by glycosidic bonds to form a ring. They are characterized by their toroidal molecular structure with hydroxyl groups orientated

toward the outside. As a result, cyclodextrins are soluble in their aqueous environment while being able to host small hydrophobic drugs in their internal cavity to form inclusion complexes [70].

Cyclodextrins containing polycations have shown efficacy in transporting nucleic acids such as plasmid DNA and siRNA. A TfR-targeting PEGylated imidazole-modified cyclodextrin was mixed with the siRNA targeting the EWS-FLI1 fusion protein known to be a transcription factor involved in tumorigenesis. Intravenous administration of this system in mice decreased gene expression of EWS-FLI1 in tumors and decreased the growth of tumors with short-term effect [71].

3.2.3. TfR-targeted micelles

TfR-targeting micelles have demonstrated efficacy to deliver nucleic acids to tumors intravenously. Micelles made of a copolymer of poly (ethylene glycol)-poly(ethylene imine) biotin were associated to biotinylated Tf by avidin-biotin non-covalent binding [72]. This micellar formulation was demonstrated to enhance the tumor delivery of antisense oligonucleotides against the human multidrug resistance protein-1 (which plays a role in multidrug resistance) in MCF-7ADR human breast carcinoma as well as in KBV human oral epidermoid carcinoma [72].

3.2.4. TfR-targeted polymers and dendrimers

Many Tf-bearing polycationic polymers have been used to condense negatively charged DNA and to deliver it to cancer cells. Among them, polylysine and polyethylenimine (PEI) have been widely used for this purpose and both were found to be very efficient vectors for gene transfer to cancer cells. In the K-562 human erythroleukemic cell line, most of the cell population was found to express the

transfected reporter genes following intravenous treatment with the Tf-bearing polylysine polyplex [73].

Similarly, Tf-bearing PEI was used to deliver a plasmid DNA encoding for tumor necrosis factor (TNF) α, a cytokine with promising anti-cancer properties but also non-specific toxicity limiting its potential use. Systemic treatment of mice bearing either subcutaneous Neuro2a neuroblastoma, MethA fibrosarcoma or M-3-melanoma cells with this targeted formulation resulted in anti-tumor effects on the three tumor models, with complete regression of MethA tumors [74,75]. The treatment was well tolerated by the animals.

In addition to their role as drug carriers, dendrimers have also been investigated as potential vehicles for nucleic acids. Generation 5- poly (amido amine) (PAMAM) dendrimer coated with PEG was targeted to TfR by conjugation with the peptide T7. It was able to co-deliver the drug doxorubicin together with a therapeutic plasmid encoding TRAIL [76]. A synergistic efficacy was observed between the 2 therapeutic agents following intravenous administration on mice bearing subcutaneous Bel-7402 human hepatoma tumors. The TfR-targeting system inhibited 77% of tumor growth, which was much higher than that obtained with non-targeted dendriplex or the free drug, and this with a smaller dose [76].

Recently, we have also demonstrated that an intravenously administered generation 3-diaminobutyric polypropylenimine dendrimer (DAB) conjugated to Tf by cross-linking and complexed to plasmid DNA encoding TNFα led to complete disappearance of 90% of A431 human epidermoid carcinoma tumors, compared to the 40% complete tumor regression observed with the non-targeted dendriplex [77]. In another study, we used this targeted dendrimer to assess the therapeutic potential of p73, a member of the p53 family of transcription factors. We demonstrated that the

intravenous administration of Tf-bearing, p73-encoding DAB dendriplex led to a sustained inhibition of tumor growth and complete tumor suppression for 10% of A431 and B16-F10 tumors in mice [78]. The treatments with both dendriplexes were well tolerated by the animals.

CONCLUSION

A major challenge for the development of potential drugs with promising therapeutic effects resides in their inability to specifically reach the pathological site following intravenous administration, without generating secondary effects to healthy tissues.

In the past few decades, the protein transferrin has been extensively explored as a targeting molecule for the transport of drug and gene delivery systems to the brain and cancer cells. A wide range of delivery approaches highlighted in this review are currently under investigation in laboratory settings and have already led to significant improvements in the systemic delivery of transferrin-bearing therapeutic systems to their site of action. Clinical trials exploring the efficacy of Tf-bearing therapeutics are already under way. These advances therefore demonstrate that the targeting of transferrin receptor has a huge potential for the development of more specific and safer gene and drug-based therapeutics.

FUTURE PERSPECTIVE

Transferrin receptor targeting has shown promises for the delivery of nucleic acids and drugs across the blood-brain barrier and to tumors. It is a research area in full expansion, which is currently the object of intense patenting worldwide.

In the future, it is likely that researchers will continue to develop novel TfR-targeted delivery systems or other techniques to further increase the specificity of the targeting to the pathological tissues. In addition, it will probably become important to focus on how to increase the transfection efficacy to optimize the therapeutic effect of potential gene medicines. One possibility would be to develop a strategy for facilitating the release of nucleic acids from the endosome to the cytoplasm of the cells.

Although highly promising, most current research on TfR-based targeted delivery of drugs and nucleic acids is still at an early stage requiring more optimization. Some clinical trials are already under way. One of them, which has for objective to test the efficacy of a Tf-cisplatin conjugate for advanced breast cancer, has already shown highly positive results in a Phase I study [57] and will hopefully pave the way for many more trials to come.

More generally, targeting the delivery of a drug to its site of action can dramatically increase its therapeutic potential. Given the significant improvements already realized in the field of TfR targeting, it is likely that targeted delivery technologies will play a crucial role in the development of future therapeutics.

EXECUTIVE SUMMARY

Introduction:

- The use of promising novel therapeutics is limited by their inability to specifically reach the pathological site after intravenous administration, resulting in toxicity to healthy tissues.

- Transferrin has been extensively studied as a targeting moiety for drug and gene delivery system, as its receptors are overexpressed on the blood-brain barrier and on cancer cells.

Transferrin receptor-mediated delivery of therapeutic agents to the brain and to cancer cells:

- A wide range of delivery systems targeting the transferrin receptor have been developed for the delivery of nucleic acids and drugs.

- Transferrin-bearing vehicles entrapping nucleic acids and drugs have been shown to specifically deliver their cargo to their required site of action and to improve their therapeutic effects.

Conclusion:

- Transferrin-bearing delivery systems are therefore highly promising for the delivery of therapeutics to the brain and tumors *in vitro* and *in vivo*, and should be further investigated.

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REFERENCE ANNOTATIONS

Publications of special note have been highlighted as:

* of interest

** of considerable interest

** 52 Daniels TR, Bernabeu E, Rodriguez JA, *et al*. The transferrin receptor and the targeted delivery of therapeutic agents against cancer. *Biochim. Biophys. Acta* 1820, 291-317 (2012).

Very detailed description of the transferrin, its receptor and all the TfR-targeting delivery strategies attempted so far.

** 74 Kircheis R, Ostermann E, Wolschek MF, *et al*. Tumor-targeted gene delivery of tumor necrosis factor-alpha induces tumor necrosis and tumor regression without systemic toxicity. *Cancer Gene Ther.* 9, 673-680 (2002).

Demonstrates that the systemically administered Tf-bearing PEI encoding TNFα resulted in anti-tumor effects on three tumor models, with complete regression of MethA tumors.

** 77 Koppu S, Oh YJ, Edrada-Ebel R, *et al*. Tumor regression after systemic administration of a novel tumor-targeted gene delivery system carrying a therapeutic plasmid DNA. *J. Control. Release* 143, 215-221 (2010).

Demonstrates that an intravenously administered Tf-bearing DAB dendriplex encoding TNFα led to complete disappearance of 90% of A431 human epidermoid carcinoma tumors in mice.

* 47 Shi NY, Zhang Y, Zhu CN, Boado RJ, Pardridge WM. Brain-specific expression of an exogenous gene after i.v. administration. *Proc. Natl. Acad. Sci. USA* 98, 12754-12759 (2001).

Demonstrates that 8D3-conjugated PEGylated liposomes encapsulating plasmid DNA encoding β-galactosidase were able to deliver their cargo to the brain of mice following intravenous administration. When the enzyme expression was driven by a brain specific promoter, gene expression was only observed in the brain.

* 57 Head JF, Wang F, Elliott RL. Antineoplastic drugs that interfere with iron metabolism in cancer cells. *Adv. Enzyme Regul*. 37, 147-159 (1997).

Describes that a clinical trial for advanced breast cancer has shown a high response rate to the Tf-cisplatin conjugate in 4 out of 11 patients, including one complete response, with only minor side effects.

* 62 Fu JY, Zhang W, Blatchford DR, Tetley L, McConnell G, Dufès C. Novel tocotrienol-entrapping vesicles can eradicate solid tumors after intravenous administration. *J. Control Release* 154, 20-26 (2011).

Describes that intravenously administered Tf-bearing multilamellar vesicles entrapping tocotrienol led to complete tumor eradication for 40% of B16-F10 murine melanoma and 20% of A431 tumors, as well as improvement of animal survival.

* 76 Han L, Huang R, Li J, Liu S, Huang S, Jiang C. Plasmid pORF-hTRAIL and doxorubicin co-delivery targeting to tumor using peptide-conjugated polyamidoamine dendrimer. *Biomaterials* 32, 1242-1252 (2011).

Demonstrates that a TfR-targeted PEGylated PAMAM dendrimer was able to co-deliver the drug doxorubicin together with a therapeutic plasmid encoding TRAIL to tumors and to inhibit 77% of tumor growth.

DEFINED KEY TERMS

• Blood-brain barrier

The BBB is a complex interface separating the peripheral circulation from the central nervous system. It is formed by brain microvascular endothelial cells connected by tight junctions. The resulting continuous endothelial wall is further reinforced by pericytes, astrocytic endfeet and vascular nerve endings surrounding the endothelial cells. This protective structure therefore prevents the delivery of most intravenously administered drugs to the brain [27].

• Avidin/biotin technology

Avidin is a glycoprotein obtained from egg white, known for its very high affinity for biotin, a vitamin that plays a role in numerous biological processes such as cell growth and the production of fatty acids. The avidin-biotin complex is the strongest known interaction between a ligand and a protein in nature, and has been shown to be resistant to extreme pH and temperature conditions [25].

• Stealth liposomes

Stealth liposomes are grafted with a polymer (generally PEG), to prevent detection by the reticulo-endothelial system. This strategy therefore increases the circulating time of the delivery system compared to conventional liposomes [32,44].

• Dendrigraft

A dendrigraft is a dendrimeric-shaped polymer formed by stepwise conjugation of tree-shaped branches on a central core.

• **Dendriplex**

A dendriplex is a complex of nucleic acids with a dendrimer.

FIGURE

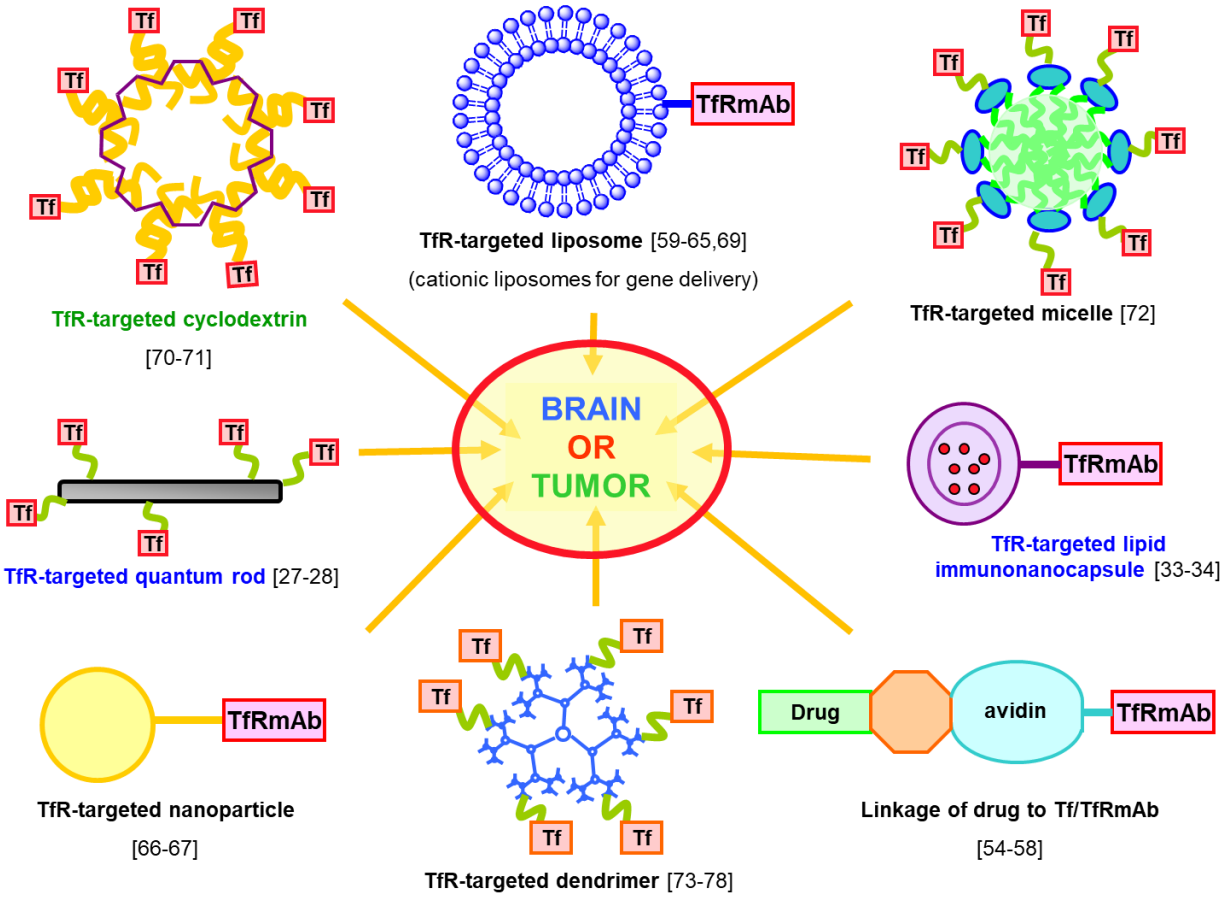

Figure 1. Examples of formulation strategies used for the delivery of drugs and nucleic acids to the brain and tumor ("Tf": transferrin, "TfRmAb": monoclonal antibody anti-transferrin receptor. Formulation name in blue: delivery to the brain, in green: delivery to the tumor, in black: delivery to either brain or tumor).

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