Review

Drug delivery via the transferrin receptor-mediated endocytosis pathway

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Abstract: The membrane transferrin receptor-mediated endocytosis has been exploited for developing novel targeted drug delivery systems, which could have a variety of applications in the site-specific delivery of anticancer drugs, proteins and therapeutic genes into proliferating malignant cells that overexpress the transferrin receptors. This is achieved by coupling transferrin or monoclonal antibody to transferrin receptor with therapeutic drugs or drug delivery vesicles. The transferrin conjugates can be obtained by use of either bifunctional chemical linkers or by genetic infusion of therapeutic peptides/proteins into the structure of transferrin / and monoclonal antibody to transferrin receptor. A variety of drug carriers such as liposomes, nanoparticles and DNA-polymer complexes (i.e. polyplex and lipoplex) were used to efficiently deliver the transferrin conjugates. Use of transferrin conjugates results in improvement in drug efficacy, selectivity and drug release as well as reduction in drug toxicity. This paper reviews the basic biochemistry of transferrin and the transferrin receptor as well as the strategy for developing targeted drug delivery system.
Keywords: Transferrin; Transferrin receptor; Drug delivery

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Contents

1. Introduction

The rapid progress of current pharmaceutical drug discovery has resulted in the emergence of increasing numbers of novel therapeutic drugs, especially biological drugs, for the treatment of a variety of diseases. However, one major problem associated with systemic drug administration is the lack of specific affinity of a drug toward a pathological site. Polymer-based and liposome-based delivery systems have the potential as specific and target-oriented delivery systems[1,2] to enhance the therapeutic index of drugs. However, delivery systems ultilizing the mechanism of receptor-mediated endocytosis (RME) are becoming an attractive option to improve the permeability, retention and target specificity of macromolecular drugs. The level of transferrin (Tf) receptor expression on the tumor cells or other abmormal cells is higher than that on the normal cells. The transferrin/Tf receptor pathway has been shown to be of great potential in the delivery of anticancer drugs, proteins and therapeutic genes into proliferating primary malignant cells[3–5].

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2. Transferrin receptor-mediated endocytosis

2.1. Transferrins

Transferrins are single-chain glycoproteins containing 700 amino acids (80 kDa). The sequence identity between different species and different members of the family is extremely high. For example, there is 78% identity between rabbit and human serum transferrin, 60% between serum transferrin and lacoferrin, and 40% identity between melanotransferrin and other transferrins. A variety of crystal structures of transferrins are available and exhibit great similarity[6]. The polypeptide chain is folded into two structurally similar but functionally different lobes, referred to N- and C-lobe, respectively. Each lobe has two domains enclosing a deep hydrophilic cleft bearing an iron-binding site. 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[7], thus Tf has been implicated in the transportation of other metal ions. Figure 1 shows the crystal structure of the transferrin and transferrin receptor complex.

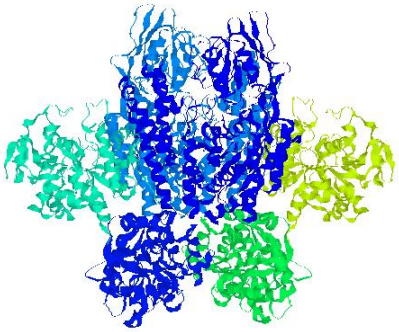

Figure 1. X-ray crystal structure of the transferrin receptor and transferrin complex. The graph was created by using RasMol (Version 2.7.0) to illustrate structural characteristics of the transferrin receptor and transferrin complex.

2.2. Transferrin receptors

There are two major types of transferrin receptors. The transferrin receptor 1 (TfR1) appears to be expressed in all nucleated cells in the body[8]. TfR1 is over expressed on rapidly dividing cells[9]. In contrast, expression of TfR1 is low or frequently undetectable in non-proliferating cells, The TfR1 is a transmembrane homo-dimer that can bind up to two molecules of Tf. The binding affinity of Tf for various receptors is very high (10^5 to 10^{10} M^{-1})[6]. Crystallographic studies of the ectodomain of human TfR1 (residues 122 to 760) revealed that the homo-dimer of TfR1 is organized as a butterfly-like shape, as shown in Figure 2.

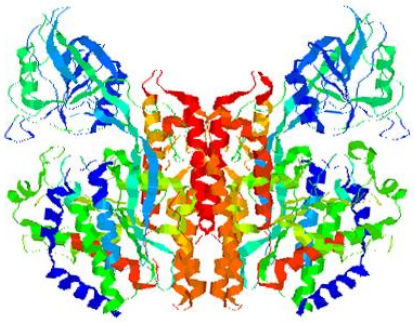

Figure 2. X-ray crystal structure of the ectodomain of the transferrin receptor 1. The graph was created by using RasMol (Version 2.7.0) to illustrate structural characteristics of the transferrin receptor 1.

The transferrin receptor 2 (TfR2) share a 45% identity and 66% similarity in its extracellular domain with TfR1. The 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 mechanism, probably related to the cell cycle or cellular proliferation status[10]. The high level of TfR2 expression in the liver suggests TfR2 possibly contributes to the liver's ability to regulate iron homeostasis[11,12]. The affinity of TfR2 for iron-loaded Tf is 25-fold lower than that of TfR1 for Tf[13].

2.3. Transferrin receptor-mediated endocytosis and iron uptake

Transferrin receptor-mediated uptake of transferrin-bound iron is one of the best-understood processes in cell biology[3,14]. Briefly, the process is triggered by the binding of Fe_2-Tf to a specific cell-surface TfR1. After endocytosis via clathrin-coated pits, which bud from the plasma membrane as membrane-bound vesicles or endosomes, the Fe_2-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. 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. Fe^{2+} is subsequently transported out of the endosome by the divalent metal transporter DMT1. After release of iron, the resultant apo-Tf: TfR1 complex is then recruited through exocytic vesicles back to the cell surface. At extracellular physiological pH of 7.4, apo-Tf dissociates from its receptor due to its low affinity at this pH and is released into the circulation, and reutilized.

3. Drug delivery based-on transferrin receptor-mediated endocytosis

Since TfR is upregulated in rapidly dividing cells, it provides the opportunity to use it for tumor-targeted delivery. Therefore, transferrin and anti-TfR antibody have long been explored for development of delivery systems for anticancer agents and genes[15], and also to transfer drugs across a variety of biological barriers via transferrin-mediated transcytosis pathways. Transferrin based drug delivery system can achieve site specific delivery of anticancer agents and circumvent multi-drug resistance, etc.

3.1. Transferrin as a metallodrug mediator

In human serum, the transferrins are primarily iron-binding proteins. Because Tf is only about 30% saturated with iron, there is still potential capacity for binding to other metal ions present in the body. Indeed, over 30 metal ions have been reported to bind to Tf. Such binding may play an important role in the transport and delivery of medical diagnostic radioisotopes such as $^{67}Ga^{3+}$ and $^{111}In^{3+}$ [16,17] and therapeutic metal ions such as Bi^{3+} [18], Ru^{3+} [19] and Ti^{4+} [20].

3.2. Transferrin conjugates in site-specific drug delivery

There are many ways to prepare transferrin conjugates. A simple method is the direct coupling of therapeutic agents to Tf by a covalent bond. When the therapeutic agents are biological molecules, incorporation of the drug into the structure of Tf using recombinant protein engineering may be of great merit[21]. Moreover, the biotin-avidin system can be a universal strategy to establish the transferrin-drug conjugates for site targeting[22]. 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 about 89 d. Although the avidin/biotin bond is stable in the circulation, it is labile at tissue depot sites[23]. Therefore, the avidin/biotin system is ideal for drug delivery to tissues.

To overcome the problem that the native transferrin in circulation may compete with transferrin-drug conjugates, coupling of therapeutic agents to anti-TfR antibody (or its Fab fragment) has been frequently employed for preparation of the conjugates[5].

3.2.1. Transferrin-drug conjugates

Many transferrin conjugates of various therapeutic agents have been prepared for specific drug delivery (Table 1)[22,24–37]. Artemisinin tagged to transferrin via carbohydrate chain has been shown to have high potency and specificity against cancer cells[38]. Doxorubicin (Adriamycin®) is an effective and widely used cancer chemotherapeutic agent; however, cardiotoxicity and emergence of resistance significantly limit its utility in clinical practice. Significantly, Tf-doxorubicin conjugate was 4–5 times more potent than free drug in doxorubicin sensitive tumor cell

Table 1. Conjugates of therapeutic agents with transferrin

Therapeutics	Linker strategy	Biological evaluation	Special remarks	References
Doxorubicin	Maleimide spacer Glutaraldehyde	MDA-MB-468 and LXFL 592	Amide or acid-sensitive link	24
	Liposome-PEG	K562	Mechanistic studies	25
		Patients	Enhanced cytotoxicity	26
	Polymeric		Encapsulated in Tf-liposome-PEG	27
	Chitosan vesicles	Mouse	Superior *in vivo* safety profile	28
	Tf-PEG-Liposome	Mouse	Circumvent P-gp-mediated MDR of tumors	29
Toxin		Tumor-bearing mice	Chloroquine block the toxicity Interstital microinfusion	30
CRM107		Rat bearing U251MG Patients		31
Insulin	Disulfide	Caco-2 and diabetic rat	A slow but prolonged hypoglyce-mic effect & dose-dependent	32
Oxaliplatin	Tf-PEG-liposome	Mousel	Enhanced extravasation of liposomes into tumors	33
Chlorambucil	Maleimide spacer	MCF7; MOLT4	Ester or acid-sensitive link; inhibit growth of tumor cells	34
γ-IFN	Liposome-PLL	MBT2	Tf-PLL promotes delivery and enhances antiproliferative activity	35
Paclitaxel	Tf-PEG-nanoparticles	Tumor bearing mice	Paclitaxel release profiles displayed a sustained release phase	36
			Biodegradable nanoparticles	37
Yeast FCU1	Antibody-avidin (Av) Fusion protein	Myeloma	FCU1 converts non-toxic prodrug 5-fluorocytosine to 5-fluorouracil	22

$F(ab')_2$, anti-tetanus fragments; MMC, mitomycin C.

lines such as HL60, Hep2 *in vitro* and 5 and 10 times more potent in resistant cell lines[39]. For highly multidrug-resistant cells, the conjugate inhibited the cell proliferation with IC_{50} of 0.025–0.2 mM, while doxorubicin did not exert any cytotoxicity even at 1 mM concentration[40].

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 3 to 18-fold enhancement of activity in the MCF7 mammary carcinoma and MOLT4 leukemia cell line, but this conjugate can be administrated at higher doses compared with unbound chlorambucil[34].

Tf-CRM107 is a conjugate protein of diphtheria toxin with a point mutation (CRM107) linked by a thioester bond to human transferrin. This conjugate exhibits potent *in vitro* cytotoxicity against mammalian cells expressing the transferrin receptor with activity at picomolar concentrations[41].

3.2.2. Transferrin and transferrin antibody coupled liposomes/nanoparticles

Liposome and nanoparticle are potent and popular carrier systems for controlled drug release. Modification of their surface with transferrin or anti-TfR antibody was shown to improve the stability, prolong the period of drug release, and most importantly increase the target specificity and drug permeability. Therefore, vast amounts of work have focused on the development of this actively targetable particle/liposome. A small unilamellar mercaptoundecahydrododecaborate (BSH)-encapsulated, transferrin-conjugated polyethyleneglycol liposomes (Tf-PEG liposomes) was prepared[42]. Intravenous injection of Tf-PEG liposomes was shown to increase the tumor retention of the drugs. Studies on the tissue selectivity of liposomes coated with transferrin-coupled polyethylene glycol revealed that Tf-dependent uptake in liver and brain was dependent on the size of the liposomes used[43]. Actively targetable nanoparticles (ATN) and PEG-coated biodegradable polycyanoacrylate nanoparticles (PEG-nanoparticles) conjugated to transferrin were developed for paclitaxel delivery[36]. The efficacy of paclitaxel-loaded biodegradable nanoparticles on tumor inhibition was determined in human prostate cancer cell line (PC3) and in a murine model of prostate cancer[37]. The IC_{50} of the drug with Tf-conjugated nanoparticles (Tx-NPs-Tf) was about 5-fold lower than that with unconjugated form or the drug in solution. Animals that received a single-dose intratumoral injection of Tx-NPs-Tf (Tx dose = 4 mg/kg) demonstrated complete tumor regression and a greater survival rate than those that received either Tx-NPs or Tx-Cremophor EL formulation. Linear and cyclodextrin-based polymers were complexed with DNAyzme molecules to form sub-50 nm particles termed 'polyplexes'[44]. These transferrin-modified nanoparticles were employed for targeted delivery of RNA-cleaving DNAzyme (short catalytic single-stranded DNA molecules) to tumor tissue. DNAzymes packaged in polyplex formulations were taken up by tumor cells upon intravenous bolus injection. The polyplex formulations could be concentrated and retained in tumor tissue and other organs, whereas unformulated DNAzyme was eliminated from the body within 24 h post-injection.

After being taken up by the cells, the transferrin-drug conjugates should follow intracellular pathways of receptor-mediated endocytosis. To optimize drug delivery, control of intracellular trafficking was studied. A pH-sensitive fusogenic peptide, GALA (WEAALAEALAEALAEHLAEALAEALEALAA) was introduced to transferrin-modified liposomes[45]. When GALA was introduced into liposomal membranes, the encapsulated rhodamines were efficiently released and diffused into the cytosol. Cross-linking of TfR induced by oligomeric Tf binding was also shown to alter the intracellular trafficking of Tf-TfR complexes[46]. Methotrexate was conjugated to Tf-oligomer and it maintain specificity of the TfR-binding, however, cross-linking of TfR redirects Tf-oligomers out of the recycling pathway, and targets them to intracellular degradation in cultured tumor cells.

3.3. Transferrin in gene delivery

The TfR-mediated gene delivery has an attractive feature since it provides an opportunity to achieve cell specific delivery of DNA complex that might also enhance the transfection efficiency. HVJ (hemagglutinating virus of Japan; Sendai virus) containing the F-transferrin chimeric protein vector demonstrated 32-fold greater tumor-targeting efficiency than wild-type HVJ envelope (HVJ-E) vector[47].

Since DNA is a large polyanionic molecule, many different polycationic molecules have been used for

this purpose, including polyornithine, histones, polyethyleneimine. Polycationic molecules and DNA can form stable molecular complexes ('polyplexes'), which are then conjugated with a cell-binding ligand transferrin or OX26 mAb to achieve receptor-mediated endocytosis.

Transferrin-polyethylene glycol-polyethylenimine/DNA complex is prepared for systemic tumor-targeted gene transfer[48]. However, polylysine is the most widely used polycation since it is available in a large variety of molecular weights and can also be easily degraded by cells[49]. 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[50].

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 non-viral vectors for the intracellular delivery of reporter or therapeutic genes in culture and *in vivo*[51]. Association of Tf with lipoplexes significantly overcame the inhibitory effect of serum and facilitated efficient transfection in many cell lines, including HeLa, K562 cells and lung carcinoma cells Calu3, H292 cells[52,53]. The structure and formation process of this efficient and efficacious Tf-lipoplex has been elucidated[54].

In addition to polyplexes-transferrin conjugate and lipoplexes-transferrin conjugate, other vectors have included coupling of genes to biotinylated Tf via streptavidin bridge[55]. There is no size limitation for the gene to be conjugated and the conjugates constructed in such a way are stoichiometrically controllable and were shown to have the potential in *in vivo* gene delivery. Gene delivery systems based on the use of Tf modified nanoparticales have also been developed[56].

However, Tf trancytosis gene delivery still suffers from a lower efficiency compared with viral vectors. Future work needs to focus on enhancing transfection efficiency that may include synthesis of novel bifunctional carriers such as inorganic nanorods, chemically modifying the system, such as optimizing parameters affecting surface binding and association and developing a specific mechanism to effectively release therapeutic genes from the endosome into the cytosol.

3.4. Other applications

The transferrin conjugate drug delivery systems are also applied to improve drug delivery across blood brain barrier (BBB) and improve the absorption of drugs by oral administration or via the pulmonary route. Insulin-transferrin conjugate was developed for treatment of diabetes. It was shown that oral administration of insulin-transferrin conjugate permeates across the caco-2 cells through transferrin receptor-mediated transcytosis and exhibit hypoglycemic effect in diabetic rats[57]. The possibility of using TfR-mediated transcytosis for systemic delivery of therapeutic proteins via the alveolar epithelium was examined using cultured primary rat alveolar epithelial cell monolayers[58]. A nanogel encapsulated oligonucleotides with surface modified with transferrin can be effectively transported across the BBB through the transcellular pathway in an *in vitro* model[59] .

4. Summary

In the past few decades, intensive studies have been made toward understanding Tf trafficking and Tf/TfR mediated cellular iron uptake pathway. The strategy of exploiting Tf/TfR as a drug carrier/delivery system is based on elevated levels of transferrin receptors present on the surface of tumor cells. The Tf/TfR delivery system also increases the permeability across a variety of biological barriers, e.g. BBB and intestinal mucosa. 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. A vast number of Tf-conjugated systems for delivering therapeutic drugs/genes including small molecule anticancer drugs, peptides or proteins have been developed. Results indicate that the well-tunable stoichiometric properties and stability of the conjugates by using different linkage strategies make the targeted drug delivery feasible via the Tf-mediated endocytosis pathway.

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转铁蛋白–转铁蛋白受体系统在药物运输中的应用

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摘要: 膜转铁蛋白受体介导的内吞作用应用于新的药物靶向给药系统, 包括将抗肿瘤药物, 蛋白和治疗基因定位运输至恶性增殖细胞或是中枢神经系统。通过偶联转铁蛋白或其抗体连接到转铁蛋白受体上成为治疗的药物或转运载体。多种药物载体如脂质体, 纳米颗粒与DNA复合物用于高效运输治疗物。转铁蛋白复合物的运用不仅提高了药效, 改善了选择性与药物释放性能, 降低了毒性, 同时也延长药物血浆半衰期, 提高药物主动靶性。本篇综述介绍了转铁蛋白/转铁蛋白受体介导的药物运输的研究进展。

关键词: 转铁蛋白; 转铁蛋白受体; 药物运输