Transferrin: structure, function and potential therapeutic actions

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There are many proteins that can multi-task. Transferrin, widely known as an iron-binding protein, is one such example of a multi-tasking protein. In this review, the multiple biological actions of transferrin, including its growth and cytoprotective activities, are discussed with the view of highlighting the potential therapeutic applications of this protein.

The transferrin (Tf) protein contains 679 amino acid residues and has a molecular weight of ~79 kD [1]. The molecule is stabilized by 19 intra-chain disulfide bonds and is protected by three carbohydrate side chains of which two are N-linked (Asn-413 and Asn-611) and the third is O-linked (Ser-32) (Swiss-Prot P02787, http://us.expasy.org/sprot/). The Tf molecule is divided into two evolutionary related lobes, designated the N-lobe (336 amino acids) and C-lobe (343 amino acids), which are linked by a short spacer sequence. Each lobe contains two domains comprising a series of α-helices, which overlay a central β-sheet backbone (Figure 1). The domains interact to form a deep, hydrophilic metal ion-binding site. The binding site in both the N- and C-terminal lobes has four conserved amino acids including two tyrosines, one aspartic acid and one histidine (N-terminal lobe – Asp-63, Tyr-95, Tyr-188 and His-249). These residues are arranged in a distorted octahedral arrangement [2]. In addition, the binding site requires two further oxygen molecules donated by a carbonate molecule to stabilize the iron atom [3]. The surrounding amino acid residues (Gly-65, Glu-83, Tyr-85, Arg-124, Lys-206, Ser-248 and Lys-296 on the N-terminal lobe) are thought to further stabilize the metal-binding site, and they have crucial roles in iron release [2]. For example, it has been demonstrated that Lys-206 and Lys-296, which are located on opposite domains of the N-terminal lobe, are hydrogen bonded to one another in the 'closed' iron-bound form. When the pH is reduced, this hydrogen bond is broken allowing the domains to rotate forming an 'open' conformation which promotes iron release [4].

Transferrin polymorphism

Tf has several polymorphisms with >30 different species detected to date [5]. There are, however, three major isotypes known as B, C and D. The majority of people carry the C allele, in particular C1, whereas the D allele predominates in parts of southwestern Africa [6].

Several studies have shown a link between Tf polymorphism and susceptibility to disease. These include the rare autosomal recessive disorder atransferrinemia [7], cardiovascular disease (CVD) [8] and Alzheimer's disease [9]. Individuals possessing the Tf C2 allele in combination with the C282Y allele of the haemochromatosis (HFE) gene have a higher risk of developing Alzheimer's disease. This risk is further increased where the individual also contained the apolipoprotein E epsilon 4 (Apo E4) allele. The authors of this study concluded that the presence of these protein variants might lead to an excess of redox-active iron resulting in oxidative damage to the neurones. By contrast, studies examining the prevalence of Alzheimer's disease in the Korean population were unable to establish a link with the Tf C2 variant [10].

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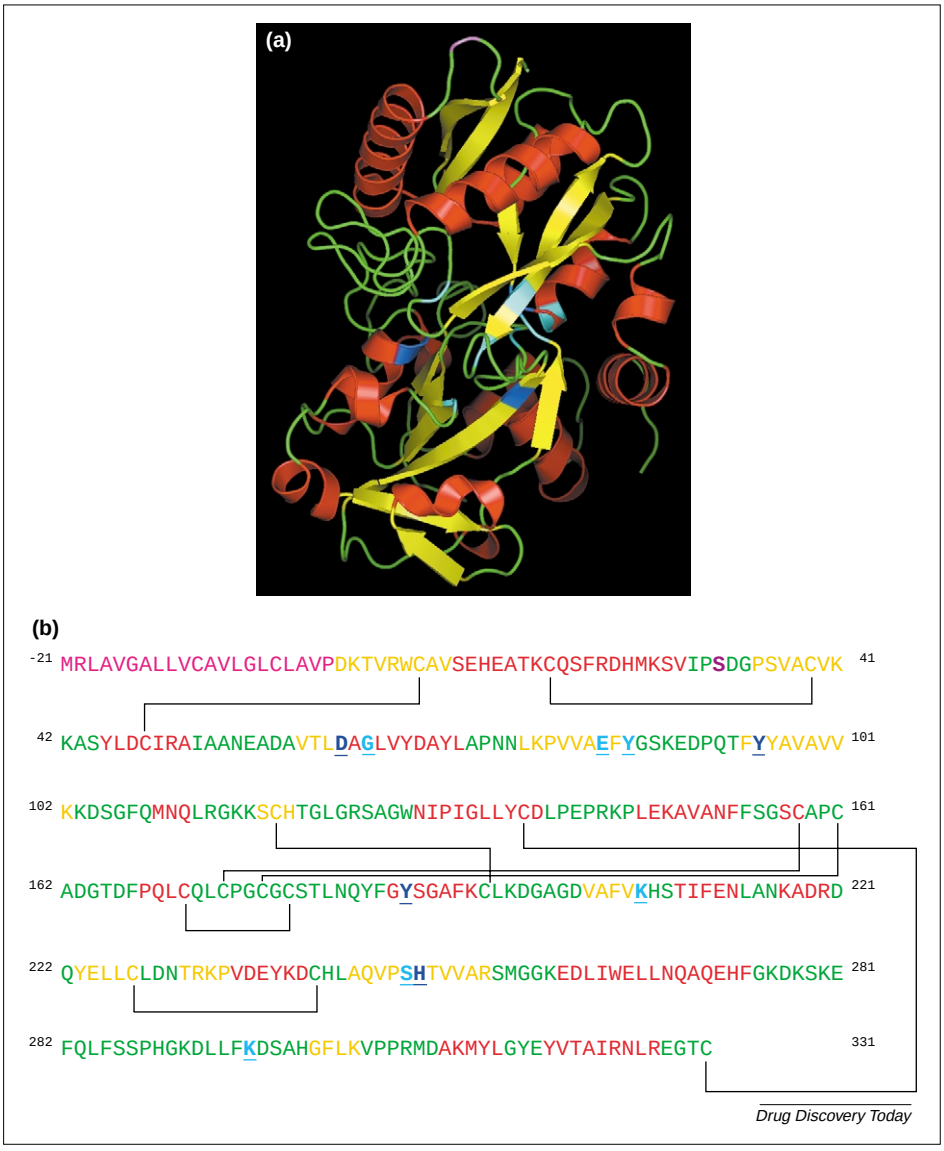

FIGURE 1

The structure and sequence of transferrin. (a) X-ray crystal structure of transferrin [Protein Data Bank – 1a8e (http://www.rcsb.org/pdb/)]. The yellow arrows indicate the ß-sheets. **(b)** Amino acid sequence of the N-terminal lobe of transferrin [2]. The iron-binding site residues are underlined and highlighted in dark blue, and the amino acids involved in stabilizing the metal-binding site are underlined and highlighted in light blue. Key: pink, leader sequence; yellow, ß-sheet; red, α-helix; green, random coil; violet, O-linked carbohydrate attachment at Ser-32. The disulfide bonds linkages are marked in solid black lines.

Protein distribution

Tf is synthesized predominantly by hepatocytes [7]. Other tissues expressing Tf include Sertoli [11,12], ependymal [13], oligodendroglial [14], metastatic melanoma cell lines [15] and human breast cancer cell lines [16]. Tf has been detected in various body fluids including plasma, bile, amniotic, cerebrospinal, lymph and breast milk [17]. Plasma concentration of Tf is stable from birth, ranging from 2 g l^{-1} to 3 g l^{-1}, and the *in vivo* half-life of this protein is eight days [18]. The level of Tf is important for healthy growth with levels below 0.1 g l^{-1} associated with an increased incidence of infection, growth retardation and anemia [19].

Protein function

Iron binding

Iron is important for all living organisms. It plays a central role in DNA replication with one of the key enzymes, ribonucleotide reductase, requiring iron as a co-factor. Iron also acts as a co-factor for heme [20]. However, free iron can be toxic, promoting free radical formation via the Fenton and Haber–Weiss reactions, thus resulting in oxidative damage to tissues [18]. Free iron causes lipid peroxidation by converting hydroperoxides into reactive peroxyl and alkoxyl radicals [21]. In addition, Tf promotes auto-oxidation reactions involving carbohydrate aldehyde groups and protein amino groups, which results in the formation of glycated products [22]. For these reasons, it is vital that iron is transported in a redox-inactive form. The primary role of Tf is therefore to transport iron safely around the body to supply growing cells.

The binding and release of iron by Tf involves several factors, for example, pH, temperature, chelator and ionic concentrations [4]. Although a carbonate ion is crucial for stabilizing the iron-binding site, other anionic species such as chloride ions are also involved in maintaining the functionality of the binding site. He *et al.* demonstrated that chloride ions retard iron release at a neutral pH but, under acidic conditions, the presence of chloride ions accelerates the release of iron [4]. Therefore, the authors concluded that iron binding and release are controlled by a combined pH–anion mechanism.

Transferrin–transferrin receptor system

Iron-loaded Tf binds transferrin receptors (TfR) on the surface of actively dividing cells [23]. Subsequently, the Tf–TfR complex is internalized and transported to endosomes. ATP-dependent proton pumps then force H^+ ions into the endosomes reducing the pH to 5.5, thus promoting iron release [24]. Under low pH conditions, the TfR alters conformation to enable apo-transferrin (apo-Tf) to remain bound. The binding characteristics of the apo-Tf–TfR complex are such that the apo-Tf is released only once the complex reaches the cell surface [23]. The Tf molecule then circulates until it again comes in contact with free iron at intestinal sites and hemoglobin breakdown (e.g. macrophages) sites, and the cycle of Tf-mediated iron redistribution is continued. It has been estimated that one Tf molecule could participate in this transport cycle as many as 100 times [25] (Figure 2).

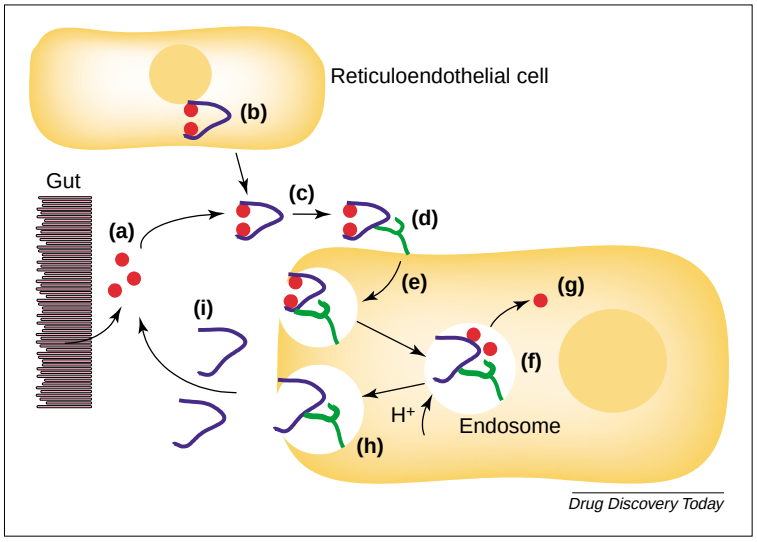

FIGURE 2

Iron uptake of cells via transferrin–transferrin receptor pathway. (a) Iron (red dots) enters the bloodstream from the gut and/or from the breakdown of hemoglobin in reticuloendothelial cells **(b)**. **(c)** Apo-Tf (indicated in blue) binds iron, which then induces a conformational change to a closed structure. **(d)** Monoferric or diferric Tf binds to the TfR (indicated in green) and the Tf–TfR complex is internalized into an endosome **(e)**. **(f)** The ATP-dependent protein pump lowers the pH of the endosome to 5.5, thus facilitating iron release. **(g)** Iron binds to a low-molecular-weight carrier molecule, which assists delivery to various intracellular locations including mitochondria (heme biosynthesis) and ferritin (storage). **(h)** The receptor-bound apo-Tf returns to the cell surface where the neutral pH promotes release of apo-Tf into the circulation. **(i)** The apo-Tf is released into the circulation. Abbreviations: Apo-Tf, apo-transferrin; Tf, transferrin; TfR, transferrin receptor.

Tf binds to at least two distinct types of TfRs, designated TfR1 and TfR2 [23]. TfR1 is expressed on a range of cells, including red blood cells, erythroid cells, hepatocytes, monocytes and the blood–brain barrier. TfR2 is expressed as two transcripts (α-TfR2 and β-TfR2), with α-TfR2 expressed predominantly on liver cells and β-TfR2 expressed at low levels on a variety of cell types [26]. The level of TfR expression varies depending on the cell type. Non-dividing cells can have extremely low levels of TfR expression, whereas rapidly proliferating cells (e.g. carcinoma cell lines) can express up to 100 000 TfRs per cell [16].

Antimicrobial activity

A further consequence of the high levels of free iron in the body is that it promotes the growth of pathogens [27]. In humans, bacterial pathogens can sequester free iron by: (i) releasing low-molecular-weight siderophores; (ii) actively uptaking heme; or (iii) binding holo-transferrin via TfR-like receptors [1]. Pathologies promoting high levels of free iron are therefore often associated with an increased incidence of bacterial infection, for example, hereditary and secondary haemochromatosis, liver failure, cardiovascular surgery, premature birth and hematological malignancies [1,28]. Parkkinen *et al.* examined the ability of apo-Tf to sequester free iron as a means of reducing infection [29]. The antimicrobial activity of apo-Tf might not just be limited to simply reducing free iron levels, and studies have identified apo-Tf as being capable of reducing the adhesion of gram-positive and -negative bacteria to surfaces [30].

Growth, differentiation and cytoprotection

Tf has been implicated in growth and differentiation activities including myotrophic [31], embryo-morphogenic [32], proliferative [33], mitogenic [34], neurotrophic [35], chemotactic [36] and angiogenic activities [36]. These effects are believed to be at least partially independent of the iron-binding role of Tf because apo-Tf preparations have been shown to possess growth-promoting effects [37]. Furthermore, Tf has been suggested to have paracrine and autocrine roles. For example, hypertrophic chondrocytes can synthesize large amounts of Tf and, along with other undifferentiated cells, express TfR on their cell surfaces [38]. This paracrine action of Tf has also been taken advantage of by some cancer cell lines; in particular, the proliferation of brain melanoma metastasis is promoted by Tf produced by brain cells [39].

It is important to recognize that Tf is just one of many growth factors combining to modulate cell growth and differentiation. Each cell type will respond to different combinations of stimulatory and inhibitory signals. Further, the cell response to these signals changes throughout the life cycle of the cell. This is illustrated in rats where intracranial injections of apo-Tf in two- to seven-day-old rats results in rapid differentiation of oligodendroglial cells. By contrast, injections in ten-day-old rats do not cause differentiation in these cells [37]. Similarly, Tf has been implicated in the growth of human colon tumor cell lines [40]. Again, the role of Tf is dependent on the level of differentiation. Studies involving less-differentiated HCT116 cells show that Tf exposure causes increased epidermal growth factor (EGF) binding. By contrast, exposure of Tf to more-differentiated colon cancer cell lines (e.g. CBS and GEO) has the reverse effect, reducing EGF binding [41].

Iron-bound Tf has been shown to inhibit apoptosis in ovarian cancer cells [42]. The apoptopic pathway, involving *Myc* activation, Fas ligand (FasL), tumor necrosis factor α (TNF-α) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), acts by upregulating ferritin which results in reduced levels of intracellular iron. The presence of iron-bound Tf is, however, able to restore the iron levels in the cell, thereby preventing cell death. Similarly, Tf has been reported to protect lymphohemopoietic and hepatic cells from Fas-mediated cell death [43,44]. The ability of Tf to modulate cellular events, like apoptosis, probably involves additional pathways. An example was provided by Weinzimer *et al*, who demonstrated that the anti-apoptotic and anti-proliferative actions of Tf are related to its ability to bind insulin-like growth factor-binding protein 3 (IGFBP-3) [45]. When Tf is bound to IGFBP-3, the molecule loses its ability to induce proliferation of bladder smooth muscle cells and apoptosis in prostate cancer cells.

TABLE 1

Potential therapeutic applications of transferrin

Pathophysiological condition	Therapeutic application	Mode of action	Refs
Free iron and/or iron overload	Atransferrinemia	Transferrin replacement	[7,19]
	Ischemia reperfusion injury	Anti-oxidative	[50]
	Cardiovascular disease	Anti-oxidative	[18]
	Radiotherapy	Iron sequestering	[57]
	Bone marrow transplantation	Antimicrobial	[61,62]
Tumor or cancer	Targeted drug delivery	Delivery of therapeutic metals, proteins, drugs and genes via transferrin–transferrin receptor pathway	[20,17]
Growth and differentiation	Cancer therapy	Promote cytotoxicity and proliferation of lymphokine-activated killer and natural killer cells	[80]

Clinical applications

The multiple actions of Tf can be exploited to produce a range of potential therapeutic applications (Table 1).

Atransferrinemia

Atransferrinemia is a rare condition, first described by Heilmeyer *et al* in a seven-year-old girl [46]. This condition is characterized by anemia, iron overload, growth retardation and increased incidence of infection [7]. The hereditary form has been described in nine individuals from seven families [7]. Non-hereditary forms have also been reported and involve anti-Tf immunoglobulin G (IgG) [47], nephrotic syndrome [48] and erythroleukemia [49].

Infusion of apo-Tf can be used to treat the condition. In Japan, two patients received doses of 1–2 g of pure apo-Tf every three to four months for four to seven years. These individuals had normal hemoglobin levels and did not develop antibodies to Tf, indicating that treatment of juvenile hypo-transferrinemic patients (plasma levels ≤0.2 g l^{-1}) with apo-Tf can reduce growth retardation and other effects associated with the condition [19].

Ischemia reperfusion injury

Ischemia reperfusion (IR) injury is a condition that promotes oxidative stress resulting in inflammation and, ultimately, cell death by apoptosis and necrosis [50]. IR injury is associated with conditions such as stroke [51], CVD [52], renal failure [53,54] and organ transplant [55].

Because free iron is able to catalyze the production of oxygen free-radicals, the presence of increased concentrations of redox-reactive iron could potentiate IR injury. Evidence to support this hypothesis is provided by studies showing that the use of the iron chelator, deferoxamine, is protective in IR injury [56]. Hence, a potential therapy would be to use apo-Tf to scavenge free redox-reactive iron. De Vries and co-workers demonstrated in Swiss male mice that apo-Tf could decrease free-iron levels and reduce renal IR injury [56]. The apo-Tf was able to inhibit neutrophil chemotaxis and complement activation, and prevent loss of renal function in a dose-dependent manner. By contrast, iron-loaded Tf promoted cell apoptosis, was unable to inhibit neutrophil chemotaxis at the injury site and could not maintain renal function. De Vries *et al*. concluded that apo-Tf could be used in cases of acute renal failure and post-operatively after the transplant of ischemic organs.

Cardiovascular disease

Van Campenhout *et al*. has recently reported that low levels of Tf (<2 g l^{-1}) and glycation of amino residues on Tf can enhance the pro-oxidative effects of iron [18]. They argue that these effects are significant causes underlying lipid peroxidation and increase the risk of CVD in diabetes patients. Tf is a negative acute-phase protein that is down-regulated in inflammatory conditions such as diabetes [57]. Oxidative damage and neuropathy observed in some diabetes patients lead to increased loss of Tf: Tf levels in Type I diabetes patients are typically 10% lower than those in normal individuals [18]. Added to this, the glycation of Tf and hemoglobin induced by the higher glucose levels can impair iron binding and promote even higher levels of free iron in the body [58]. Thus, intravenous administration of apo-Tf in diabetic patients to minimize free iron will probably provide a means to control the oxidative damage and to reduce the frequency of CVD in these patients. Further advantages to this approach include a reduction in the Tf iron saturation level that can improve the binding of glycated forms of Tf to iron [18,59].

In non-diabetic patients, high levels of Tf iron saturation (>55%) in combination with elevated levels of low-density lipoprotein (LDL) is associated with progression of CVD [8]. This is promoted further in individuals that combine high levels of Tf iron saturation with a high intake of iron. Again, Tf in combination with other LDL-lowering therapies could be used to maintain low levels of Tf iron saturation, thereby minimizing the ability of free iron to promote LDL oxidation and limit the progression of CVD.

Radiotherapy

Levels of Tf have been shown to decrease during radiotherapy treatment [57], which has been suggested to promote oxidative stress by increasing the levels of redox-reactive iron in the circulation [57]. Thus, infusion of apo-Tf might bind the iron released during irradiation and

thereby minimize oxidative damage. Benefits of apo-Tf treatment have recently been reported in radiotherapy experiments in mice [60]. Intraperitoneal injections of apo-Tf at ~100 mg kg^{-1}, one day before irradiation, increased the number of spleen colony-forming units [60]. It was speculated that the positive effect of apo-Tf was a result of its ability to reduce free-iron levels, or via other mechanisms such as direct stimulation of bone marrow cells or modifying actions on cyclic nucleotides. The conclusion that the positive effect of Tf might involve mechanisms other than iron binding is supported by previous mouse irradiation studies where both Tf and Tf-derived glycans were shown to protect bone marrow cells [43]. The authors deduced that Tf was able to mediate this cytoprotective affect by modulating cytokine activity.

Antimicrobial activity in bone marrow transplantation patients

Bacterial infections are often responsible for the death of patients undergoing bone marrow transplantation or chemotherapy for hematological malignancies [61]. These patients typically have high levels of free iron present in the circulation, which often exceeds the binding capacity of Tf [62]. Von Bonsdorff *et al*. showed that the growth of the opportunistic pathogen *Staphylococcus epidermidis* was inhibited by normal serum [29]. However, the addition of free iron abolished the growth inhibitory effects of serum. Therefore, reducing the free-iron levels is thought to be important in preventing growth of pathogens [1]. One possible mode of treatment is intravenous infusion of apo-Tf, which has been recently tested for treatment of *S. epidermidis* infections in myeloablative and allogenic stem cell transplantation patients [29]. The authors of this study had previously demonstrated that infusion of apo-Tf at 100 mg kg^{-1} in stem cell transplant patients resulted in reduced saturation levels of Tf iron and of free iron [63]. This effect remained for 12–48 h in the majority of patients. Therefore, repeated apo-Tf injections were advocated to eliminate free iron and iron-mediated toxicity in myeloablative therapy.

Targeted drug delivery

The mechanism of iron transport and uptake via the Tf-TfR transport system has the potential to be exploited for site-specific delivery of various therapeutic metal ions, drugs, proteins and genes [17]. Of particular interest are cells that overexpress TfR [20].

Tf is normally only 30% saturated with iron in the body. At least 30 other metal ions can also bind to Tf [64]. Therefore, it is possible to use Tf to transport other metals around the body, in particular, gallium (Ga^{3+}) and indium (In^{3+}) can be transported by Tf. The cellular uptake of Ga^{3+} occurs mainly via the Tf–TfR mechanism and thus it concentrates in tissues expressing high levels of TfR, such as tumors [20,65]. It is for this reason that $^{67}Ga^{3+}$, a low-energy gamma-emitting radionuclide, has widespread use as a diagnostic technique for many malignancies [20]. $^{111}In^{3+}$ has attracted interest when used either in combination with chemotherapy for the treatment of neoplasms or as a radiolabel to determine tumor cell viability [20]. Although the $^{111}In^{3+}$ radioisotope is able to bind Tf strongly, it is not translocated into the cell [66]. This is because Tf does not release $^{111}In^{3+}$ during passage through the endosome [67]. Tf can also bind other therapeutic metals such as bismuth, boron, titanium, technetium and ruthenium. Bismuth has been used to treat syphilis, hypertension, infections, skin conditions and gastrointestinal disorders [68]. Boron contained within Tf-conjugated liposomes has been delivered to cancer cells for boron neutron-capture therapy [69]. Both ruthenium and titanium possess anti-cancer activities, and technetium has been used in tumor imaging [70–72].

Targeted drug delivery using the Tf-TfR pathway is not restricted to metal ions. Tf can also be conjugated with drugs, proteins and genes [17], for example, the diphtheria toxin [73]. The conjugated diphtheria toxin, known as Tf-CRM107 (TransMID, Xenova Group), has been infused intra-tumorally into patients with malignant brain tumors. Phase I and II trials have provided encouraging results with positive anti-tumor effects observed in patients with malignant brain tumors that are resistant to conventional therapy [74]. A Phase III trial is now under way which aims to compare the efficacy of Tf-CRM107 with that of the best standard chemotherapy in treating patients with progressive and/or recurrent glioblastoma multiforme. Other examples of anti-cancer compounds that have been used in conjunction with Tf include doxorubicin, chlorambucil and paclitaxel [75–77]. The Tf-TfR pathway can also be used to deliver small peptides by incorporating the target peptide into the primary sequence of Tf [78]. In order for this delivery system to be effective, the Tf molecule must remain able to bind the TfR, and the peptide needs to be surface orientated. Delivery of therapeutic genes via viral vectors can also be achieved using the Tf-TfR system [79].

Cancer therapy

There is some evidence to suggest that apo-Tf can be used in the treatment of cancer. Tf in combination with other factors [e.g. insulin-like growth factor 1 (IGF-1) and interleukin 2 (IL-2)] can promote cytotoxicity and proliferation in lymphokine-activated killer (LAK) cells and natural killer (NK) cells. Okamoto *et al*. developed a specific Tf-containing cell growth media (RDSF) that can stimulate both growth and proliferation of these LAK cells [80]. In the presence of IGF-1, Tf was crucial for cell proliferation, which suggests that the media could be used in adoptive immunotherapy for cancer.

The iron-binding ability of Tf has been used in conjunction with the anti-malarial drug, artemisinin (ART), to improve drug resistance in human small-cell lung carcinoma (SCLC) [81]. ART is a naturally occurring compound that, in the presence of Fe^{2+}, breaks down into a toxic

compound. The study showed that ART was able to kill the SCLC cells at nanomolar concentrations after pre-treatment with Tf. In a similar manner, Efferth *et al*. have demonstrated that leukemia cells are also more effectively killed when ART compounds are used in conjunction with Tf [82]. The authors identified higher levels of TfR expression in the leukemia cells (45–95%) when compared with those expressed by the normal mononuclear blood cells (0.4–1.3%). Hence, the combination treatment was postulated to target the cancer cells more effectively.

Conclusions

Tf, like many plasma proteins (e.g. vitamin D-binding protein) [83], has evolved additional functions apart from its primary activity of binding and transporting iron. From a therapeutic perspective, this enables the one molecule to be used for various treatments. This review has highlighted some of these potential therapies – using Tf to sequester free iron, to deliver drugs to rapidly growing cells, to activate immune cells and to prevent cell apoptosis.

Tf, being an abundant plasma protein, is an ideal candidate for purification from plasma [84]. Recombinant forms might also be manufactured to alter the metal-binding site or by inserting peptide sequences. For example, Tyr-188 has been replaced with a phenylalanine to alter the binding affinity from iron to copper [85]. In this way, delivery systems could be tailored for specific delivery of drugs to rapidly dividing cells. Tf is therefore an ideal candidate molecule for companies wishing to pursue new therapeutic options.

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