Review

The membrane-bound transferrin homologue melanotransferrin: roles other than iron transport?

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Abstract Melanotransferrin (MTf) is a membrane-bound transferrin (Tf) homologue that is found at high levels in melanoma cells. MTf has many characteristics in common with serum Tf and previous studies have shown that it can bind Fe. This has led to speculation that MTf may be involved in Fe transport. Because Fe is required for a variety of metabolic reactions including ATP and DNA synthesis, MTf could play a role in proliferation. However, recently it has been shown that MTf plays very little role in Fe uptake by melanoma cells, and unlike other Fe transport molecules (e.g. the transferrin receptor), its expression is not controlled by Fe. In the present review the function of MTf is discussed in relation to data suggesting other roles apart from Fe uptake.

Key words: Iron; Melanoma; Melanotransferrin; Transferrin; Transferrin receptor

1. General introduction

Iron (Fe) plays a crucial role in cellular metabolism as it is essential for a wide variety of metabolic reactions upon which life depends, including ATP and DNA synthesis. Indeed, it has been suggested that Fe was an essential component for the genesis of life (for review see [1]). Due to this vital requirement for Fe, cells developed a repertoire of Fe-binding and transport proteins known as the transferrins [1]. The most well known of these proteins is serum transferrin (Tf) which is involved in Fe transport in the plasma. This molecule donates its Fe to cells via its specific binding to Tf receptors (TfRs) present on the cell membrane (Fig. 1) [1]. The Tf is then internalised by receptor-mediated endocytosis, the Fe is released from the protein within an endosome, and then transported into the cell to become part of the intracellular Fe pool. The Fe may be used for a variety of crucial enzymes such as ribonucleotide reductase or haem proteins and stored in ferritin (Fig. 1) [1].

It was of great interest that in the early 1980s the first Tf-like molecule was discovered [2–4]. This protein was initially known as human melanoma antigen p97 since it was first identified at high levels in malignant melanoma cells [2–4]. It was later called melanotransferrin due to its high homology with Tf. The properties shared between human MTf and Tf include: (i) it has 37–39% sequence homology with human serum Tf, human lactoferrin, and chicken Tf; (ii) the MTf gene is on chromosome 3, as are those for Tf and the TfR; (iii) many of the disulphide bonds present in serum Tf and lactoferrin are also present in MTf; (iv) MTf has an N-terminal Fe-binding site that is very similar to that found in serum Tf, while the C-terminal is different and does not bind Fe; and (v) isolated and purified MTf can bind Fe from Fe(III)–citrate complexes [2–7]. These observations appeared to indicate that MTf plays a role in Fe transport.

The most obvious difference between MTf and serum Tf is that Tf circulates in the plasma, while MTf is bound to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor [8,9]. Indeed, MTf can be removed specifically from the cell membrane using phosphatidylinositol-specific phospholipase C (PI-PLC [8–10]). Apart from the membrane-anchored form, MTf can also be secreted, and this is not just due to passive partitioning from the membrane into the medium [8,9]. The function of secretory MTf remains unclear and largely unexplored.

2. Can human melanotransferrin transport iron?

Initial studies in the early 1990s examined the relative roles of MTf compared to the TfR in Fe uptake by the human melanoma cell line SK-Mel-28 [11–15]. This cell line was used because it expresses the highest levels of MTf of all cell types tested ($3–3.8\times10^5$ MTf sites/cell [2]). The SK-Mel-28 melanoma cell incorporates Fe from Tf by two processes consistent with receptor-mediated endocytosis and pinocytosis of Tf [11,12,15]. In addition, these cells take up Fe from low-M_r Fe complexes by a TfR-independent process [13]. Of interest, a membrane-bound, protease-sensitive, Fe-binding component was identified in SK-Mel-28 cells consistent with MTf [11–13]. However, while this membrane Fe-binding component could bind Fe, it did not appear to donate it to the cell [12,13].

Further studies using Chinese hamster ovary (CHO) cells transfected with *MTf* [10] showed that this molecule could transport Fe derived from ^{59}Fe–citrate complexes but not Tf. However, the levels of MTf (1.2×10^6 sites/cell [10]) in transfected cells were 3–4-fold greater than that found on the SK-Mel-28 cell line ($3–3.8\times10^5$ sites/cell [2]). Because Fe

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Abbreviations: CHO, Chinese hamster ovary; GPI, glycosyl phosphatidylinositol; MTf, melanotransferrin; PI-PLC, phosphatidylinositol phospholipase C; pICA, porcine inhibitor of carbonic anhydrase; Tf, transferrin; TfR, transferrin receptor

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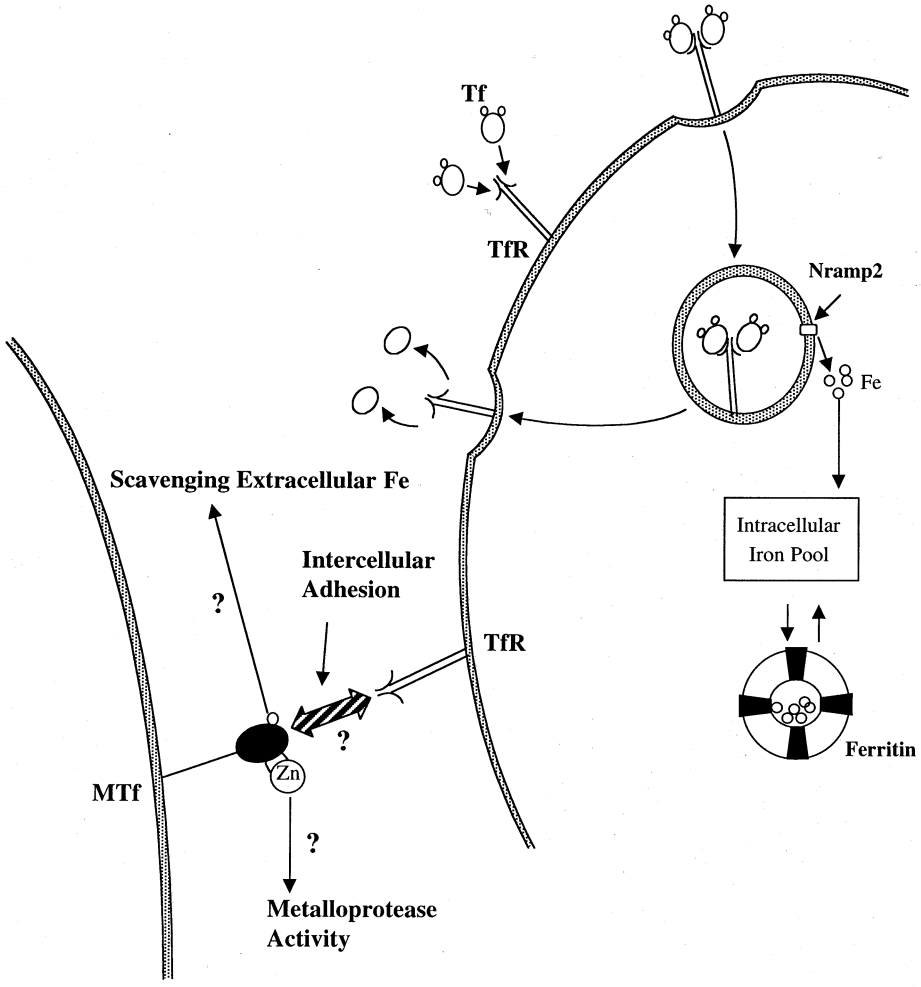

Fig. 1. Schematic illustration demonstrating the role of the Fe-binding protein Tf in Fe uptake and the possible roles of MTf. The Tf in the serum binds to the TfR on the cell membrane which is then internalised by receptor-mediated endocytosis. Once the Fe is released from Tf it passes through the endosomal membrane by the Fe transporter Nramp2. The Fe then enters a poorly characterised compartment known as the intracellular Fe pool. Iron in the pool is used for the synthesis of important Fe-containing proteins or can be stored in the Fe-storage protein ferritin (see Section 1). MTf is found bound to the cell surface by a phosphatidylinositol anchor and does not play a significant role in Fe uptake by melanoma cells where it is expressed at very high levels (see Section 2). The possible functions of MTf may include: (1) prevention of free radical production and membrane damage by acting as an Fe scavenger, (2) cell surface metalloprotease, or (3) intercellular adhesion (via docking with the TfR on other cell types) (see Section 5).

uptake from ^{59}Fe–citrate by MTf-transfected CHO cells was only 2.4-fold that of control CHO cells after a 4 h label [10], these data questioned the role of MTf in the Fe uptake process of melanoma cells where it was expressed at lower levels [1].

Considering these latter results, further experiments were designed to characterise the pathophysiological role of MTf in Fe uptake by the SK-Mel-28 melanoma cell. Initial studies examined if modulation of intracellular Fe levels using the Fe chelator desferrioxamine (DFO) or the Fe source ferric ammonium citrate (FAC) could change *MTf* mRNA levels. Under conditions of Fe depletion, up-regulation of the TfR increases Fe uptake from Tf which corrects the deficiency, while down-regulation of TfR levels occurs when intracellular Fe concentration increases [1]. In contrast to TfR mRNA that increases after exposure to DFO and decreases after incubation with FAC, there was no change in *MTf* mRNA levels. In addition, compared to control cells, there was no alteration of ^{125}I-labelled anti-MTf monoclonal antibody (mAb) binding in cells exposed to DFO or FAC, suggesting no change in the number of MTf sites. Further studies examined the ability of DFO and FAC to modulate Fe uptake from ^{59}Fe–citrate which is bound by MTf [3,10,13]. In contrast to the expected effect of DFO or FAC at increasing and decreasing Fe uptake from ^{59}Fe–Tf respectively [1], DFO had no influence on ^{59}Fe–citrate uptake, whereas FAC markedly increased it. Collectively, these studies suggest that MTf was not regulated like the TfR in response to cellular Fe levels [16]. Indeed, if MTf were a crucial transporter of Fe for cellular metabolism, it may be expected that its regulation would be comparable to the TfR [16]. Regulation of Fe uptake is essential as Fe is vital for cellular metabolism and its depletion can lead to death. On the other hand, Fe overload can be toxic. These data demonstrating no change in MTf expression after manipulation of intracellular Fe levels are supported by previous immunohistochemical studies in the liver, showing that MTf expression did not alter in Fe overload disease in contrast to Tf and the TfR [17].

As described above, MTf can be removed from the membrane by PI-PLC [8–10]. Preincubation of melanoma cells with PI-PLC reduced anti-MTf mAb binding to 3% of the control, while PI-PLC only slightly reduced ^{59}Fe uptake from ^{59}Fe–citrate [16]. These results suggest that MTf played little role in Fe uptake from ^{59}Fe–citrate by these cells [16]. Using a similar experimental protocol, more recent studies have also demonstrated that depletion of MTf from HeLa cell membranes had little effect on Fe uptake from Fe–citrate [18].

The results demonstrating first that MTf is not regulated by Fe like the TfR, and second that PI-PLC treatment does not lead to a marked decrease in Fe uptake from Fe–citrate, suggest that MTf may not play a major role in Fe uptake by these cells [16]. Indeed, it was previously shown that SK-Mel-28 cells avidly take up Fe from Tf [11,15]. Moreover, MTf is not involved in Fe uptake from Tf [10,13], which is the major form of Fe in the circulation (for reviews see [1]).

Other evidence suggesting that MTf is not crucial for Fe uptake include that it is not found in all melanoma cell lines, varying from 0.3 to 80% of that found for SK-Mel-28 melanoma cells [19]. In addition, MTf expression has not been consistently found on other rapidly growing cancer cells or normal tissues [20]. Furthermore, while TfR numbers increase prior to DNA synthesis [42], due to the Fe requirement of ribonucleotide reductase [21], MTf density remains constant throughout the cell cycle [22]. Seligman et al. [23] have shown by mAb-binding studies in HL-60 leukemic cells and FAMC 110 melanoma cells that there is no change in MTf expression between confluent cells, growing cells, or cells grown in high Tf concentrations. Therefore, these data again suggest that MTf is not vital for obtaining Fe [1,12,13]. It cannot be completely discounted that in contrast to neoplastic cells, MTf in normal resting cells could play some role in Fe internalisation. Indeed, MTf could act as an Fe scavenger to inhibit membrane lipid peroxidation and this is discussed further in Section 6.1. However, more work is required to investigate this possibility.

MTf was initially described as an oncofoetal antigen because it was found at high levels in tumours and foetal tissues and was either not expressed or only slightly expressed in normal tissues [19,24]. More recently, there have been reports of human MTf being identified in normal tissues, including sweat gland ducts [8,25], liver endothelial cells [8,17], and the endothelium and reactive microglia of the brain [26,27]. In addition, normal sera contained very low levels of MTf [28]. The expression of *MTf* mRNA (poly(A)$^+$) has also been examined amongst 50 human tissues and was found to be markedly different than *Tf* mRNA or *TfR* mRNA [16]. Surprisingly, *MTf* mRNA expression was widespread in normal tissues, and was observed at its highest levels in the salivary gland. In contrast to expectations, *MTf* mRNA expression was generally greater in adult rather than foetal tissues [16], which is different to that previously reported [19,24,28]. Last, the high expression of MTf in the proximal kidney tubule [29], salivary gland [16], and sweat gland ducts [8,25] argues against a role in Fe transport, since Fe is never actively excreted and remains tightly bound to Tf [30].

Considering all of the data collectively, it could be suggested that MTf may play additional roles separate to its ability to bind Fe.

3. Melanotransferrin and the brain

There have been some data indicating that MTf may play a role in the brain. In a number of reports Jefferies and associates have shown that reactive microglia associated with the amyloid plaques in brains of patients with Alzheimer's disease express MTf [26,31]. From these results it has been suggested that the increased levels of MTf in the cerebrospinal fluid and serum of Alzheimer's disease patients [32] originate from the reactive microglia associated with the senile plaques [31]. Apart from the reactive microglia, MTf was also expressed in the capillary endothelium of the brain and had a distribution similar to that found for the TfR [26,27]. The role of MTf in the brain remains unclear and further studies are essential in order to define its function.

4. Other physiological roles of transferrin homologues

Apart from MTf, other Tf homologues have also been identified with physiological roles unrelated to Fe transport. These include saxiphilin (a neurotoxin inhibitor), pacifastin (a proteinase inhibitor) and the porcine inhibitor of carbonic anhydrase (pICA). The Tf homologue saxiphilin from the bullfrog *Rana catesbeiana* contains two Tf lobes with high homology to serum Tf (51% homology to frog serum Tf). However, this molecule does not have Fe-binding activity, as most of the critical Fe-coordinating amino acids are not conserved [33]. Instead of binding Fe, saxiphilin binds saxitoxin, a neurotoxin produced by various cyanobacteria and dinoflagellates [33].

The heavy chain of the protein pacifastin from the plasma of crayfish, *Pacifastacus leniusculus*, is a Tf homologue that acts as a proteinase inhibitor [34]. Pacifastin contain two covalently linked subunits, a heavy chain which is the Tf homologue, and a light chain with proteinase inhibitory activity. The proteinase inhibitory effects of pacifastin require the Tf heavy chain for activity [34]. Interestingly, the heavy chain retains its ability to bind Fe, and it has 33% sequence homology with cockroach Tf and human MTf. However, no evidence has been presented to indicate that pacifastin plays a role in serum Fe transport [34].

The porcine Tf homologue pICA (65% homology with porcine serum Tf) is found in serum and functions as an inhibitor of carbonic anhydrase (CA) [35]. The ability of this molecule to inhibit carbonic anhydrase appears to be its main function, with little evidence of an Fe-transport role [35]. Despite the high degree of similarity, pICA is functionally and biochemically different from the Tfs. All other Tfs tested failed to bind or inhibit CA and they are antigenically distinct [35]. Hence, considering these examples and the lack of any Fe transport role of MTf in melanoma cells, it would not be unexpected that MTf plays other biological roles.

At this point it is of interest to note that the polypeptide motifs essential for binding metals apart from Fe show similar evolutionary trends to other functions. For example, the heavy chain of mammalian inter α-trypsin inhibitor has significant similarity to the multicopper-binding domain in the group of multicopper oxidase proteins [36]. It can be speculated that the evolution of proteins capable of binding essential metal ions for metabolism and growth in simple organisms may have preceded other proteins with more specialised roles. Perhaps the existing scaffolding provided by the genetic

Table 1
Comparison of the iron- and anion-coordinating amino acids present in MTfs, MTf homologues and other transferrins

Protein	Source	N-terminal repeat (position of each amino acid)	C-terminal repeat (position of each amino acid)
Melanotransferrins	Human	**DYRYH** (78,107,136,210,279)[a]	**SYSYH** (421,451,482,556,625)
	Chicken	**DYRYR** (77,106,135,209,278)	**GYRYQ** (420,450,481,555,624)
	Rabbit	**DYRYH** (78,107,136,210,279)	**NYSDH** (421,451,482,556,625)
	Mouse	**DYRYH** (78,107,136,210,279)	**RYSYH** (421,451,482,556,625)
	Drosophila CG10620	**DYSYD** (93,121,153,231,295)	**DYTHN** (505,533,563,640,709)
	Drosophila CG3666	**TYLYP** (85,114,144,228,294)	**DYGDG** (426,453,483,529,597)
Transferrins			
Serum transferrin[b]	Human	**DYRYH** (82,114,143,207,268)	**DYRYH** (411,445,475,536,604)
Lactotransferrin[c]	Human	**DYRYH** (80,112,140,212,273)	**DYRYH** (415,455,485,548,617)
Transferrin	Cockroach	**DYRYQ** (78,111,141,225,295)	**DYRYH** (429,457,487,573,642)

GenBank accession numbers (protein ID): human MTf – NP_005920; chicken MTf – CAA63003; rabbit – BAA33956; mouse – BAA86655; *Drosophila* (CG10620) – AAF49900; *Drosophila* (CG3666) – AAF58039; human serum Tf – NP_001054; human Lf – NP_002334; cockroach Tf – A47275.
[a]In parentheses are positions of each iron-coordinating amino acid in the protein sequence.
[b]Invariant in human, rabbit, bovine, porcine, equine and chicken serum transferrin.
[c]Invariant in human, bovine and murine lactoferrin.

code of primitive metal-binding proteins provided a convenient framework for building other molecules.

With regard to possible other functional roles for MTf separate from Fe uptake, it is relevant to discuss that protein sequence conservation in different organisms does not always translate into the same physiological role in eukaryote metal metabolism. This phenomenon is observed in the *Arabidopsis* Fe transporter Irt1p, where its conserved counterpart in the yeast is a Zn transporter, ZRT1 [37]. Similarly, the human Nramp2 Fe transporter (also known as the divalent metal ion transporter, DMT1) only shows strong homology to yeast manganese transporters Smf1p and Smf2p, and not the yeast Ftr1p (high-affinity) or Fet4p (low-affinity) Fe transporters [38]. These data suggest that conserved metal binding and sequence similarity of shared polypeptide motifs are not always a sufficient determinant of physiological role.

5. Melanotransferrin homologues – clues to functions apart from iron transport?

To date MTf has been identified in the rabbit (GenBank accession number AB010995), mouse (GenBank AB024336), and chicken (GenBank X91908), with no evidence to suggest high-affinity Fe binding and transport as a primary function. Indeed, in contrast to human, mouse, and rabbit MTfs that have conserved Fe-binding residues (Asp, Tyr, Arg, Tyr and His) in their N-terminal domain (but not in the C-terminus; see Table 1), chicken MTf contains a substitution that may prevent Fe binding (Table 2). Hence, chicken MTf with atypical N- and C-termini appears to have evolved away from high-affinity Fe binding [8]. Apart from this difference, human MTf and MTfs from chicken, rabbit, and mouse show several common properties including: (i) a much higher sequence identity or homology between themselves compared to other

Table 2
Comparison of the active site of putative Zn^{2+}-dependent peptidases in standard one-letter amino acid symbols and the suggested site in human and other melanotransferrins

Peptidase	Sequence	GenBank accession number Protein ID.
E-24.11 (human)	^{582}**IGHEITHGFD**^{591}	P08473
E-24.11 (rat)	^{582}**IGHEITHGFD**^{591}	P07861
E-24.11 (rabbit)	^{582}**IGHEITHGFD**^{591}	P08049
Fibroblast collagenase (human)	^{216}**AAHELGHSLG**^{225}	NP_002412
Stromelysin (human)	^{216}**AAHEIGHSLG**^{225}	AAB36942
Gelatinase (human)	^{411}**AAHEFGHAMG**^{420}	A28153
Aminopeptidase N (human)	^{386}**IAHELAHQWF**^{395}	CAA31640
Surface protease gp63 (*Leishmania*)	^{259}**VTHEMAHALG**^{268}	AAB96339
Neutral protease (*Bacillus subtilis*)	^{362}**TAHEMTHGVT**^{371}	BAA01604
Neutral protease (*Serratia* sp.)	^{174}**FTHEIGHALG**^{183}	999638
Peptidase N (*Escherichia coli*)	^{295}**IGHEYFHNWT**^{304}	AAA24317
Thermolysin (*Bacillus stearothermophilus*)	^{370}**VGHELTHAVT**^{379}	S72175
Aminopeptidase A	^{391}**VAHELVHQWF**^{400}	NP_001968
Leukotriene A4 hydrolase	^{294}**IAHEISHSWT**^{303}	NP_000886
TRH-degrading enzyme	^{438}**IVHEICHQWF**^{447}	NP_037513
Neutral endopeptidase	^{582}**IGHEITHGFD**^{591}	NP_009220
Angiotensin-converting enzyme	^{412}**AHHEMGHIQY**^{421}	P22966
Human MTf	^{344}**LGHEYLHAMK**^{353}	NP_005920
Mouse MTf	^{344}**LGQEYLQAMK**^{353}	BAA86655
Chicken MTf	^{343}**LGDEYLHGMQ**^{352}	CAA63003
Rabbit MTf	^{344}**LGPEYLHAMK**^{353}	BAA33956

Tf family members; (ii) the conservation of all 28 cysteine residues suggesting similar protein folding and tertiary structure; (iii) all four MTfs are anchored to the plasma membrane by GPI anchors [9,29,39,40].

The identification of MTf in other species has suggested a variety of functions. For example, in the mouse and rabbit, the major site of MTf expression is on the cell surface of chondrocytes, and it has been suggested that it is involved in the control of chondrocyte differentiation [39,40]. Similarly, chicken MTf may influence eosinophil differentiation, as the gene is a direct target of the GATA-1 and C/EBPβ transcription factors that are responsible for the differentiation of eosinophils [29].

Recently the genome of *Drosophila melanogaster* has been sequenced [41], and two genes were identified with sequences indicating they are homologues of human MTf [41]. Based upon the homology between vertebrate MTf and the fly homologues, the authors describe that these molecules could mediate the main Fe uptake pathway in the fly [41]. The article states that there is conservation of the residues in these two proteins that are essential for Fe binding, and it is concluded that the fly MTfs appear to be a ancestral mechanism involved in Fe uptake [41].

However, comparing the fly MTf sequences to the consensus Fe-binding residues required for Fe binding in the Tf family, the critical Fe coordinating amino acids DYRYH [42,43] were not conserved (Table 1). For instance, in the fly MTf-like molecule (CG10620; GenBank AAF49900), there are two changes in the consensus Fe-binding sequences in the N-terminus (R to S and H to D) and three changes in the consensus Fe-binding sequences in the C-terminus (R to T, Y to H, and H to N; Table 1). In the second fly MTf (CG3666; GenBank AAF58039), there are three changes in the consensus Fe-binding sequences in the N-terminus (D to T, R to L and H to P) and three changes in the consensus Fe-binding sequences in the C-terminus (R to G, Y to D and H to D; Table 1). These changes are important, as minor alterations in the critical Fe-coordinating residues can markedly influence Fe binding [42,43]. For example, the C-terminal domain of human MTf is unable to bind Fe because of changes in two of the five Fe-binding residues [7]. Considering these changes, it is unlikely that the fly MTfs would mediate Fe uptake in the same way as Tfs with consensus Fe-binding domains. Indeed, even with a conserved N-terminal Fe-binding site, MTf plays little role in Fe uptake by melanoma cells [16] where it is expressed at very high levels [2,3].

The high conservation of MTf but not its Fe-binding sites in organisms with diverse evolutionary backgrounds again suggests that this molecule plays an important role separate from its ability to bind Fe. The possible biological roles of MTf are discussed below.

6. Possible functional roles of melanotransferrin

The three sections below discuss possible roles of MTf based upon preliminary evidence. Obviously, further investigation is essential in terms of clearly defining its biological role.

6.1. Protection against membrane-lipid peroxidation

Discussing multiple roles of Tf homologues, it is of interest to note that serum Tf itself plays a number of roles in the organism. For instance, it is the main protein involved in Fe transport in the plasma, and in relation to this, it also has a protective function via its binding of low-M_r Fe which prevents toxic free radical production [1,30]. Through its roles of binding Fe, Tf also has a bacteriostatic function against some microbes, as Fe is essential for their growth [30]. If MTf has a protective role, and considering that it is membrane-bound, it may act as a capture and hold mechanism to prevent toxic free Fe deposition on membranes. Alternatively, MTf may limit the presence of excessive amounts of free Fe released from damaged tissues. In some pathological situations where there is cell death, Fe is probably released, and MTf may act to bind this and prevent lipid peroxidation Indeed, MTf has an Fe-binding site that is very similar to that found in Tf and can bind Fe from low-M_r complexes such as Fe(III)–citrate [3,4,7]. Considering this, while previous studies have demonstrated that MTf does not play a significant role in Fe internalisation by melanoma cells, a membrane-bound Fe-binding component was identified that was consistent with MTf [11,12].

6.2. Metalloprotease activity

Elegant molecular modelling studies have demonstrated that a potential Zn(II)-binding thermolysin consensus sequence in MTf is oriented in an appropriate stereochemical arrangement to allow it to bind Zn(II) that is crucial for metalloprotease activity [44]. Indeed, the Zn-binding site responsible for catalytic activity in thermolysin is nearly superimposable on the MTf site [44]. Examining the MTf molecules found in a variety of species, only human MTf contains the consensus sequence **HEXXH** found in metallopeptidases such as thermolysin (Table 2). This was a surprising finding, and could suggest that MTf found in other species could have functions apart from metalloprotease activity.

6.3. Intercellular adhesion molecule

The high homology of MTf with serum Tf [4] suggests that it could bind to the TfR. Indeed, there has been a preliminary unrefereed conference report indicating that isolated MTf can bind to the TfR [45]. Considering these data, it is important to determine whether this interaction may be important in terms of intercellular adhesion. For example, MTf bound on one cell could interact with the TfR on another, effectively acting as a 'key and lock' mechanism and aiding intercellular docking. It can be hypothesised that this docking may target MTf metalloprotease action to the sites of invasion. Indeed, such a protease-docking function would not be unusual, as integrins have been proposed to act in this way, forming invadopodia [46].

7. Conclusions

Membrane-bound MTf does not play a significant role in the uptake of Fe from low-M_r Fe complexes or Tf, suggesting that it may have other unexpected roles. Indeed, this is suggested by its different tissue distribution to Tf and the TfR that is the major Fe uptake pathway. Considering other roles of MTf, it is of interest that the Tf molecule appears to be an ancient but useful scaffold upon which to build a number of different proteins with a variety of functions. While the role of MTf remains speculative at present, further studies using

knock-out animals are essential in order to understand the role of this interesting molecule.

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