Transferrin and cellular iron exchange

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Introduction
The importance of transferrin and iron for cell growth is well illustrated by the observation that proliferating cells in vitro have an absolute requirement for transferrin [1]. Although transferrin binds iron with a very high association constant ($\sim 10^{30}$ [2]) cells are able to remove the iron and incorporate it into functional and storage compounds without degradation of the carrier protein. After removal of the iron, transferrin is released from cells and its unoccupied iron-binding sites become available to bind iron. Most of this comes from erythrocytes destroyed in the reticuloendothelial system. Smaller amounts of iron are released into the plasma by the gut mucosa, after absorption of food iron, and by the liver, where a depot of iron exists. Some of the cellular aspects of this cycle of iron exchange with transferrin have been studied using erythroid cells and in particular the reticulocyte. More recently, with the advent of techniques for the isolation and culture of non-erythroid mammalian cells it is becoming apparent that many of the underlying mechanisms in iron exchange are common to all cells. To utilize transferrin iron cells must express a plasma membrane receptor for the protein which allows the binding and uptake of transferrin and its passenger iron. The physical and chemical properties of this receptor, isolated from a variety of cells and tissues, have been studied.

Characterization of the transferrin receptor
The molecular characteristics of the transferrin receptor, isolated from the rabbit reticulocyte membrane, were first reported by Aisen and colleagues [3, 4]. The binding protein has a molecular size of 176 000 daltons, comprising two identical subunits, and, in common with other membrane receptors, contains carbohydrate residues. The dimensions of the receptor-transferrin complex in solution, $4 \text{ nm} \times 36 \text{ nm}$ [4], indicate that the receptor could easily span the cell membrane. Similar characteristics have been reported for the human transferrin receptor isolated from placenta [5] and more recently from human leukaemia cells [6, 7]. Stein & Sussman [8] have reported that the transferrin receptors from normal and malignant tissue have identical peptide maps, indicating that transformed cells express the same receptor as normal cells. The 180 000 daltons dimer probably binds two transferrin molecules [7, 9]. The function of the receptor can be destroyed by proteases but not by neuraminidase [10], suggesting that the carbohydrate moiety is not vital for transferrin binding. Partial proteolysis of the receptor on the exterior of cells and in a microsomal preparation [11] suggests that a 70 000 daltons fragment of the receptor is exposed on the cell surface whereas a 5000 daltons region is accessible at the interior of the membrane, leaving a region of approximately 15 000 daltons spanning the membrane.

Expression of transferrin receptors
Tumour cell-lines, which grow rapidly in culture, have the receptor at all stages of growth, but the receptor density may vary with the growth phase of the cell [12, 13]. In contrast, the reticulocyte rapidly loses its ability to bind transferrin as it matures to an erythrocyte [12, 14] and the peripheral blood lymphocyte only begins to synthesize the receptor after mitogen stimulation [13, 15, 16].

Quantification of the receptors expressed by whole cells usually employs a functional assay of the binding of radiolabelled transferrin to cells at temperatures between 4°C and 37°C. At the lower temperatures two- to three-fold less transferrin is bound by cells than at physiological temperatures.
transferrin bound to the surface receptor at 37°C is internalized by the cell, as judged by its uptake and release of transferrin. Since this distribution of receptors is the same as found for cell-associated transferrin at 37°C, it is probable that transferrin and its receptor remain associated during the endocytic cycle. Recently, direct evidence that this is the case has come from a preliminary report by Harding et al. [21], in which gold-labelled transferrin was observed to remain closely associated with the membrane of intracellular vesicles after uptake by cells.

The reported association constants for the binding of transferrin to cells are somewhat variable but fall within the range from $2 \times 10^7$ l/mol to $2 \times 10^8$ l/mol, depending on the temperature and conditions used [15, 16, 18, 22]. Estimates of receptor numbers vary from 40,000 per cell, on isolated rat hepatocytes [22], to between 100,000 [23] and 300,000 per cell [24], on rabbit reticulocytes. The availability of iron to cells may influence receptor numbers. Ward et al. [18] reported that receptor expression in cultured human fibroblasts bore a reciprocal relationship to the amount of inorganic iron added to the medium.

In developing erythroid cells [25] transferrin and iron uptake are closely parallel regardless of the maturity of the cells. This is probably because, owing to the high demand for iron by these haemoglobin synthesizing cells, most of the iron is stripped from the transferrin taken up. However, in cultured cells this close coupling of transferrin and iron uptake appears to break down. These cells bind as much transferrin as erythroid cells but take up iron at very low rates [12, 16, 17]. Faulk et al. [26] suggested that transferrin and malignant cells may bind large amounts of transferrin to disguise surface antigens rather than expressing the receptors to facilitate iron uptake. However, this now seems unlikely in view of more recent evidence that transferrin is endocytosed after binding to its receptor and would, therefore, be unavailable for antigen disguise on the cell surface. Furthermore, others [6, 7, 15] have found that probably all proliferating cells, including transformed lymphocytes, express transferrin receptors and that their growth has an absolute requirement for transferrin and iron in the culture medium [1]. Taken together these findings suggest that the receptors on these cells function, as they do on normal cells, to allow the uptake of iron. During infection, malignancy and inflammation serum iron falls to low levels [27] and it is under these conditions that lymphoid cells must be activated by noxious stimuli and respond by proliferating. It is possible that the enhanced expression of transferrin receptors by transformed lymphocytes enables them to grow unchecked by the prevailing iron-deficient environment. Similarly, the ability of malignant cells to proliferate under these conditions may be facilitated by their expression of large numbers of functional transferrin receptors.

Iron uptake and utilization

It appears that iron delivery to cells, at normal plasma iron saturations, will be dominated by diferric transferrin since the magnitude of the association constant for the binding of diferric transferrin to its receptor is sufficiently high to give almost complete saturation of transferrin receptors in vivo, given the usual concentration of diferric transferrin in human plasma ($\sim 4 \text{ } \mu\text{mol/l}$). Monoferri transferrins bind with a lower association constant (S. P. Young & A. Bomford, unpublished observation), which helps to explain why, although cells are able to remove the single iron atom from monoferri transferrin, it is only one-seventh as effective an iron donor as diferric transferrin when they are in competition [28]. At pH 7.4 and 37°C apotransferrin binds to the receptor with an apparent association constant (in the rat) only one-thirtieth of that of diferric transferrin [29]. Thus, even though much of plasma transferrin is in the iron-free form, it should not interfere with the process of iron uptake by cells.

Reticulocytes are able to strip both iron atoms off diferric transferrin simultaneously, showing no preference for either binding site of the protein, when taking up iron at optimal rates [30, 31]. Little preference is shown for iron borne by either of the monoferri transferrins [32]. Fletcher & Huehns [33, 34] originally proposed that the two sites of transferrin might deliver iron preferentially to different tissues and Aiwa et al. [35] provided some support in vivo for this hypothesis. However, this effect in vitro may have been caused by the use of an incomplete nutrient medium [36], undermining the suggestion that there are important functional differences between the two iron-binding sites of transferrin. Experimental
evidence for the equivalence in the function of the two iron-binding sites for iron delivery to isolated rat hepatocytes has also been reported [30]. However, these cells appear not to strip the transferrin of both iron atoms, but return some monoferric transferrin to the medium [30], suggesting that binding of transferrin to the receptor does not inevitably lead to the removal of all of its iron.

Some workers have suggested [37, 38] that transferrin remains on the cell surface, in complex with its receptor, during iron removal by reticulocytes. However, others have shown that after transferrin binds to cells at 37°C it rapidly becomes inaccessible to proteases [16, 17, 19], suggesting that it undergoes endocytosis. Electron-microscopic autoradiographic studies have demonstrated transferrin deep within the reticulocyte [10, 39], an observation extended by the localization of ferritin-labelled transferrin in intracellular vesicles, under conditions where ferritin alone was excluded [40]. The inhibitory effect of colchicine and vinblastine on transferrin uptake implies a role for the microtubules in this process [41]. More recently it has been shown that methylamine and ammonium chloride, which increase intravesicular pH, enhance transferrin uptake, but decrease the release of iron from the protein to the cells [42]. Morgan has proposed this as evidence for the involvement of lysosomes in the release of iron to the cell, since transferrin binds iron only weakly at the pHi of lysosomes. The acid environment of these organelles could easily facilitate the release of iron from the protein. However, little transferrin is degraded during the process of iron delivery [17, 43], even though it is known that the protein is susceptible to proteolysis in lysosomes [43]. With some other endocytic processes, such as the uptake of asialoglycoproteins by hepatocytes, it is apparent that the ligand passes through a pre-lysosomal, acidic compartment in which the low pH facilitates the cleavage of the ligand/receptor complex [44], thereby allowing the recycling of the unoccupied receptor to the cell surface and routing of the ligand to the lysosomes. It is known that transferrin, unlike many other ligands, remains associated with its receptor at low pH [5] and it has recently been proposed [45, 46] that it is this property which allows transferrin to escape transfer to the lysosomes. By remaining associated with its receptor transferrin is recycled to the exterior of the cell in an undegraded form. Furthermore, apotransferrin also adheres to the receptor at low pH and, if iron is removed from transferrin during its passage through the acidified vesicle, the iron-free protein would similarly escape routing to the lysosomes and be released from the cell.

Given this proposed mechanism for iron release from internalized transferrin, it might be expected that only apotransferrin would be released from the cells if all of the vesicular iron was transferred to the cell. However, it has been shown that transferrin can be released from cells carrying some iron [30, 47]. These observations suggest that the locus for the control of iron uptake by cells may be at the point at which iron is removed from internalized transferrin, rather than at the plasma membrane. As discussed earlier different cell types express different numbers of transferrin receptors, as do cultured cells at various phases in their growth cycle [12] and, although the locus for the control of iron uptake is probably intracellular, an element of control, perhaps longer term, may thus be exerted at the cell membrane by modulation of receptor numbers.

Once iron is released from transferrin the metal must be transferred to the sites of utilization. In the reticulocyte the major destination for the iron is haemoglobin, but in other cells the proportion used for haem synthesis is much less. In Chang cells [48] approximately 60% of the iron taken up from transferrin is found in ferritin, whereas in phytohaemagglutinin-stimulated human lymphocytes, incubated with transferrin for a short time (3 h), 35% was found in ferritin, only 5% in haem and the remainder in an undefined fraction (A. Bomford & S. P. Young, unpublished observation). A proportion of the last-named may represent the so-called labile or easily chelatable iron pool [49]. The role proposed for this pool is to act both as a precursor which is incorporated into functional complexes [50] and as the immediate source of iron for release from cells [51]. It is known that inorganic iron compounds may be utilized by isolated mitochondria for haem synthesis and it is possible that the free pool of iron is the immediate source of iron for mitochondria inside the cell [52]. However, Ulvik & Romslo [53] have shown that ferritin iron can also be mobilized by isolated mitochondria for haem synthesis and this form of iron has been suggested as an obligatory intermediate for haem synthesis in reticulocytes [54]. This suggestion is contradicted by recent results from Egyed [55], who showed that iron incorporated into ferritin by reticulocytes was not subsequently used for haemoglobin synthesis. Furthermore he identified several non-haem/non-ferritin complexes within this cell which could be potential sources of iron for the mitochondria. The chemistry of iron probably precludes the existence of free ionic forms of the metal under physiological conditions, and its propensity to form complexes with many intracellular proteins and low molecular weight compounds may explain
why many putative intermediates in the transport of iron have been found. It remains to be seen which of these complexes are of physiological importance.

Iron release from cells

After donation of its iron, transferrin is released from the cell and is then available for many more plasma-to-cell cycles, as is indicated by its long plasma half-life (7-10 days) compared with its passenger iron (1.5-2 h) [56]. The protein then acquires more of the metal for another transport cycle to occur and most of this comes from the cells of the reticuloendothelial system, after erythrocyte breakdown. Iron seems to be released from these cells in vivo by a process which leads to random binding of the iron to any available vacant transferrin iron-binding site, both on monoferric transferrin and apotransferrin [57]. Since dipher ferr transferrin is a better iron donor in vitro than monoferric transferrin [28] it is likely that, in vivo, transferrin must acquire two atoms of iron before it becomes an effective iron donor. Iron released from isolated rat hepatocytes also binds randomly to any empty iron-binding sites on transferrin [30]. The absence of specific receptors for apotransferrin on hepatocytes suggests that the iron-free protein acts as a passive acceptor of iron rather than monoferric transferrin [29] it is likely that, on iron availability, may play a part.

References

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